Evaluation of Different Pre-treatments of Chromium Leather Waste and Their Use in Biogas Production

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Zusammenfassung/Abstract

Deutsch

Die weltweite wirtschaftliche Bedeutung der Lederindustrie ist unumstritten, da sie ein Nebenprodukt der Fleischindustrie, die Häute, in ein Produkt mit hoher Wertschöpfung umwandelt. Allerdings werden durch die Verarbeitung von Leder jährlich tausende von Tonnen neuer fester Nebenprodukten produziert, die im Gegensatz zu Häuten eine geringere biologische Abbaubarkeit aufweisen. Üblicherweise erfolgt die Gerbung unter Einsatz basischer, dreiwertiger Chromsalze. Aufgrund der enthaltenen Schwermetalle dürfen die Gerbereinebenprodukte, wie die Falzspäne chromgegerbter Häute und Beschneideabfälle chromgegerbter Leder nicht deponiert werden. Dennoch erfolgt die Entsorgung überwiegend durch Deponierung oder Verbrennung, ungeachtet möglicher Konsequenzen für die Umwelt. Diese Nebenprodukte aus der Lederherstellung sind verantwortlich für die größten ökologischen Herausforderungen, die durch Gerbereien verursacht werden.

Da die chromhaltigen festen Gerbereinebenprodukte hauptsächlich aus Kollagen (organischen Stoffen) bestehen, ist eine Verwertung der Gerbereiabfälle zur Biogasproduktion durch den anaeroben Abbau möglich. Die größte Herausforderung ist die Stabilität des Nebenprodukts gegenüber Temperaturen von bis zu 110 ° C und enzymatischen Abbau. Ursache für diese hohe Stabilität ist die bei Kollagen typische dreidimensionale native Struktur, die durch zusätzliche chemischer Vernetzungen zwischen den Kollagenfasern durch Cr^{3+} Salze weiter stabilisiert wird. Daher werden chromhaltige feste Gerbereinebenprodukte bisher nicht industriell zur Produktion von Biogas verwendet.

Das Hauptziel dieser Arbeit ist die Beschleunigung und Verbesserung der Biogasgewinnung unter Einsatz von chromhaltigen festen Gerbereinebenprodukten als Substrat, sodass diese industriell genutzt werden können. Dazu muss die stabile Struktur zuvor denaturiert werden, um einen enzymatischen Abbau während der Biogasproduktion zu erreichen. Zu diesem Zweck wurde das Substrat durch Extrusion und hydrothermische Behandlung (kontinuierliche und als Batch) vorbehandelt. Die Charakterisierung der vorbehandelten Gerbereinebenprodukte erfolgte durch Rasterelektronenmikroskopie (SEM), Differenzkalorimetrie (DSC), Abbau durch Trypsin und Kollagenase sowie durch Messung ihrer Löslichkeit in Wasser. Anschließend wurde das Biogasproduktionspotential des vorbehandelten Substrates in Batch und in kontinuierlichen Versuchen bestimmt, um die Verwertung von vorbehandelten schwermetallhaltigen festen Gerbereinebenprodukten als Substrat zur Produktion von Biogas zu bewerten.

Die Ergebnisse zeigen, dass es möglich ist, die kollagene Struktur chromhaltiger fester Gerbereinebenprodukte durch Vorbehandlung abzubauen. Die Extrusion und hydrothermische Behandlung des Substrates in Batch-Versuchen reduzierten die lag-Phase der Biogasproduktion um vier bis fünf Tage und verringerten die Abfallmenge. Bei Batch-Versuchen mit Substratüberladung durch vorbehandeltes Material wurde teilweise eine Diauxie beobachtet. Der Zusammenhang von Diauxie und übermäßiger Produktion flüchtiger Fettsäuren wurde untersucht. Im Gegensatz zu den extrudierten Chromfalzspänen (Reste der Halbfabrikatherstellung) zeigten extrudierte Lederabfälle, welche mittels Nasszurichtung und Zurichtung in Gerbereien hergestellt wurden, trotz Vorbehandlung (Extrusion) ein schlechtes Abbauverhalten. Kontinuierliche Versuche mit vorbehandelten chromhaltigen festen Gerbereinebenprodukten lassen den Schluss zu, dass es möglich ist, eine höhere Beladungsrate zu verwenden und die tägliche Methanproduktion zu steigern. Den Ergebnissen zufolge, können vorbehandelte chromhaltige feste Gerbereinebenprodukte als Substrat zur Produktion von Biogas im industriellen Maßstab eingesetzt werden.

Schlüsselwörter: *Biogasproduktion*; *Anaerober Abbau*; *Feste Gerbereinebenprodukt*

English

The worldwide economic importance of the leather industry is undeniable, as it converts a by-product of the meat industry, the hides, into a value-added product. However, processing of leather generates thousands of tons of new solid waste annually and, unlike hides, this waste has a low biodegradability. Usually, the tanning process is carried out using basified trivalent chromium salts. As a result, a substantial part of the solid waste is chromium-tanned and normally disposed of through landfill or incineration processes, despite the possible ecological consequences. This waste is responsible for the main ecological challenges caused by tanneries.

As chromium leather waste mainly consists of collagen (organic matter), this waste can be considered for biogas production through anaerobic digestion. The main challenge to be overcome is the stability of the waste towards temperatures of up to 110 °C and enzymatic degradation. This high stability is caused by the three-dimensional native structure typical for collagen, and additional chemical cross-links between the collagen fibres achieved by Cr^{3+} salts. Therefore, hitherto chromium leather waste is not utilized industrially to produce biogas.

The main goal of this study is to accelerate and improve the biogas production process using chromium leather waste as a substrate in a way that it can be used industrially. However, to achieve enzymatic degradation this stable structure has to be denatured, otherwise, the generation of biogas is hindered. For this purpose, the following pre-treatments were performed on the chromium leather waste: extrusion, hydrothermal treatment, and autoclave. The pre-treated waste was evaluated using scanning electron microscopy (SEM), differential scanning calorimetry (DSC), degradation by trypsin and collagenase, and by measuring their solubility in water. Subsequently, the biogas production potential of the pre-treated waste was investigated using batch and

continuous trials to examine the feasibility of using pre-treated chromium leather waste as substrate to produce biogas.

Results show that it was possible to degrade the collagenous structure of the chromium leather waste through pre-treatment. Extrusion and hydrothermal treatment of the substrate in batch trials reduced the lag-phase of the biogas production by four to five days and reduced the remaining waste compared to the untreated waste. In some batches, when using a substrate overload of pre-treated waste, diauxie was observed. The relation of diauxie and excessive production of volatile fatty acids was studied. Results for extruded leather off-cuts show that the wet end and finishing process in tanneries make this material more inaccessible to degradation, even after pre-treatment. Pre-treated chromium leather waste used as substrate in continuous trials demonstrated that it was possible to use a higher loading rate and obtain a higher daily methane production. These results showed that pre-treated chromium leather waste can be used as substrate to produce biogas on industrial scale.

Keywords: *Biogas production*; *Anaerobic digestion*; *Leather waste*

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Symbols

Greek symbols

Symbol	Description	Unit
λ_1	Lag-phase	d
λ_2	Switch to the second phase of the anaerobic digestion	d
μ_m	Maximum biogas production rate	L kg^{-1} d^{-1}

Constants

Symbol	Description	Unit
e	Euler's number	-

Latin symbols

Symbol	Description	Unit
A	Biogas formation potential	L kg^{-1}
COD	Chemical oxygen demand	g L^{-1}
$Coll_0$	Onset collagen content	mg
$Coll_f$	Final collagen content	mg
D	Degradation by trypsin or collagenase	%
DD	Degradation degree of collagen	%

I	Organic dry matter of the inoculum in the reactor	g
m_0	Onset mass	mg
m_f	Final mass	mg
$m_{substrate}$	Substrate addition	g d^{-1}
p	P-value	-
R^2	Coefficient of determination	-
S	Organic dry matter of the substrate	g
SW	Solubility in water	%
t	Time	h; d
V_{Gas}	Volume of biogas	mL d^{-1}
x_{CH4}	Amount of methane	-
y	Cumulative biogas production	L kg^{-1}

List of abbreviations

A3	Shavings **a**utoclaved during **3** minutes
A6	Shavings **a**utoclaved during **6** minutes
A12	Shavings **a**utoclaved during **12** minutes
A24	Shavings **a**utoclaved during **24** minutes
A48	Shavings **a**utoclaved during **48** minutes
A96	Shavings **a**utoclaved during **96** minutes
A192	Shavings **a**utoclaved during **192** minutes
A384	Shavings **a**utoclaved during **384** minutes
ANOVA	**An**alysis **o**f **v**ariance
CDU	**C**ollagen **d**igestion **u**nits
C/N	**C**arbon to **n**itrogen ratio
CS	**C**hromium **S**havings
DSC	**D**ifferential **s**canning **c**alorimetry
E100D	Shavings **e**xtruded **d**ry at **100** °C
E130D	Shavings **e**xtruded **d**ry at **130** °C
E150D	Shavings **e**xtruded **d**ry at **150** °C
E170D	Shavings **e**xtruded **d**ry at **170** °C
E100W	Shavings **e**xtruded **w**et at **100** °C
E130W	Shavings **e**xtruded **w**et at **130** °C
E150W	Shavings **e**xtruded **w**et at **150** °C
E170W	Shavings **e**xtruded **w**et at **170** °C
EDX	**E**nergy-**d**ispersive **X**-ray spectroscopy
EO	**E**xtruded **o**ffcuts
Gly	**Gly**cin
H140	Shavings treated **h**ydrothermally at **140** °C
H150	Shavings treated **h**ydrothermally at **150** °C
H170	Shavings treated **h**ydrothermally at **170** °C

rpm	**R**evolutions **p**er **m**inute
SEM	**S**canning **e**lectron **m**icroscopy
S/I	**S**ubstrate to **i**noculum ratio
TGM	**T**wo-phase **G**ompertz **m**odel
VFA	**V**olatile **f**atty **a**cid

1 Introduction

In 2014, the manufacture of leather and related products in the European Union generated EUR 54 billion in turnover and employed 447.535 people (GROW, 2016) highlighting the importance of the leather industry in this region. The industry also plays an important role in terms of environment, since its main raw material (hides) is a by-product of the meat industry, which otherwise would be transformed into gelatin or disposed of at landfills as waste. Yet it must be emphasized that hides were completely biodegradable at the slaughterhouse but the leather-making process decreases the biodegradability of the material (Flowers, 2017).

Between 2012 and 2014 an average of 558.4 thousand tons of leather from bovine hides were produced worldwide (FAO, 2016). Europe alone is responsible for the largest part of the global leather production, about 25% (Dhayalan et al., 2007). Buljan et al. (2000) estimated the amount of solid waste produced while processing one ton of wet salted hides (Table 1.1).

Regarding the different waste shown in Table 1.1, disposal of tanned waste generated after the tanning step is most complicated. As the chromium-based tanning process is performed worldwide in about 90% of the cases (Covington, 2009), most of this waste will contain Cr^{3+} and need special handling. Currently, the chromium-tanned leather waste is mainly disposed of through landfill or incineration processes, despite the ecological consequences (Pati et al., 2014).

Increased environmental restrictions and escalating landfill costs have encouraged the leather industry to develop cleaner technologies by minimizing waste and maximizing reuse (Mu et al., 2003). Attempts have been made to replace the chromium salts used in the tanning process but the obtained chromium-free leathers entail higher costs. Furthermore, chromium-based tanning is the most

robust and cost-effective way to produce leather. It is possible to use vegetable agents in the tannery process, but those tanning agents cannot be considered more environmentally friendly than chrome tanning due to the high wastewater load and low treatability in conventional systems (IULTCS, 2018b; Trommer and Kellert, 1999). Glutaraldehyde is used as well, but the process is more complicated to perform and economically disadvantageous for tanneries (Trommer and Kellert, 1999). Therefore, the leather industry continues to face the handling and disposal problems of chromium-containing waste.

Tab. 1.1: *Quantity of solid waste produced in the leather-making process.*

Solid waste	**Quantity (kg/ton of wet salted hide processing)**
Untanned	
Trimmings	100 kg
Fleshings	300 kg
Tanned	
Unusable split	107 kg
Trimmings	20 kg
Shavings	99 kg
Dyed/finished waste	
Fibres and trimmings	7 kg
Buffing dust	1 kg
Offcuts	5 kg
Total	639 kg

Source: Buljan et al. 2000.

The reutilization of chromium leather waste is an environmentally friendly alternative that helps to reduce the amount of waste disposed of by converting it into value-added products or creating the possibility of playing a new role in energy generation or in the development of new raw materials.

The use of this waste as substrate to produce biogas through anaerobic digestion is promising due to the reduction of the final amount of waste and simultaneously the generation of renewable energy, low level of process complexity, and low cost. This process is called anaerobic digestion of organic matter the

biological treatment of which is performed in the absence of oxygen while producing biogas, a mixture of methane (55 – 70%) and carbon dioxide (30 – 45%) with traces of other gases (Deublein and Steinhauser, 2008).

Hitherto, only a few studies on the digestion of chromium leather waste were published because it is considered as complex waste (Dhayalan et al., 2007; Ferreira et al., 2010; Priebe et al., 2016; Agustini et al., 2015 and 2018). These authors consider chromium leather waste as complex waste because of its collagen content and tanning with chromium salts. These studies demonstrate that it is possible to produce biogas from this waste but, due to very long periods of time needed for digestion and low biogas yields, the method needs further research to reach industrial feasibility. Furthermore, the final biomass produced in the biogas reactor would contain chromium, which complicates its use as a fertilizer. Extraction and recycling of chromium from the final biomass produced in the reactors also needs further research.

The collagen molecule, the main component of leather, has a triple helical structure stabilized by hydrogen bonds and natural cross-links. The amount of hydrogen bonds in the structure defines the energy necessary to denature the triple helix because more hydrogen bonds need more energy to be broken down and separate the chains (Miles and Ghelashvili, 1999). This complex structure results in high resistance to heat and enzymatic degradation, and low solubility in aqueous buffers. The denaturation temperature is different for each structural level of collagen, and to transform a collagen molecule into gelatin, a temperature of about 38 °C is needed (Meyer, 2019). The denaturation temperature to denature several collagen molecules kept together by natural cross-links forming tissue is between 60 and 65 °C (Meyer, 2019). Tissue can be further stabilized with chromium salts by chemical cross-links during the tanning step in tanneries forming chromium-tanned leather (Dhayalan et al., 2007). The chromium leather has a denaturation temperature of 105 to 110 °C (Schroepfer and Meyer, 2017). Furthermore, chromium present in chromium-tanned leather could be toxic for anaerobic bacteria (Deublein and Steinhauser, 2008).

Currently, there are no biogas plants in the industry using chromium leather waste as a main substrate due to these difficulties. However, the tannery

SÜDLEDER (Rehau, Germany) already has a biogas plant in operation using their own organic waste (hair, protein, fat, and chromium-loaded sludge) to produce energy (Schuberth-Roth, 2013). This kind of initiative illustrates the interest of the industry in biogas production, nevertheless the use of a substrate as complex as chromium leather waste needs to be further developed.

Irrespective of all difficulties, leather is composed of organic matter, hence it can be used as raw material to produce biogas. This approach would become more feasible with the development of a simple and efficient method to reverse the effects of tanning (Covington, 2009).

Several different pre-treatments can be used to denature the collagen present in chromium leather waste and enable microorganisms to degrade it in order to reduce the digestion time and increase the biogas yield. The parameters of choice for the most appropriated pre-treatment are time, cost, and complexity of the process. As the collagen molecule itself has high stability and the chromium tanning process makes it more stable (Usha and Ramasami, 2000), collagen molecules are endowed with mechanical and thermal stability of the fibrous network and high stability to enzymatic degradation. Consequently, it is necessary to denature the collagen fibres to enable the enzymes to degrade this solid waste (Kanagaraj et al., 2006).

1.1 Motivation

The production of large amounts of chromium leather waste, such as chromium leather shavings and leather offcuts, cannot be avoided. Therefore it is necessary to reuse the waste generated. Using the waste for biogas production appears to be an attractive alternative but pre-treatment is necessary to denature this stable waste.

The aim of this study is to accelerate and improve the biogas production process through anaerobic digestion of chromium leather waste enabling its application in the industry. For this purpose, the chromium leather waste underwent pre-treatment using different heating and mechanical technologies, which could be

easily adjusted to industrial scale. The modifications caused in the collagen structure of this waste were evaluated using different fast in vitro methods. Finally, the biogas formation potential was investigated through biogas production trials on laboratory (batch) and pilot scale (continuous) to prove the feasibility of the considered pre-treatments.

2 State of the Art

2.1 Chromium leather waste

Chromium leather waste is collagen-based waste generated after the tanning step with chromium salts in the leather-making process. In this study, we use two kinds of chromium leather waste, chromium shavings and automotive leather offcuts. These materials were selected because both are generated in large amounts and are difficult to reuse.

Chromium shavings are generated after the tanning step. Hides are soaked, unhaired, limed, delimed, bated, and pickled. These steps are carried out to purify the hide and open up its structure in preparation for the tanning step with basified trivalent chromium salts (Covington, 2009). After tanning, tanned leather has to be shaved to adjust its thickness generating the chromium shavings (Heidemann, 1993). These are wool-like waste with 40 to 50% of water content. This waste mainly consists of the collagen of hide with complex bond Cr^{3+} salts and some residues from the tanning process (fat, salts, or mineral compounds).

Leather offcuts are finished leather leftovers that underwent more steps in the leather-making process than the chromium shavings. In addition to the tanning step, this waste was retanned, dyed, fatliquored, dried, and finished with polymer coatings (Heidemann, 1993). Those are necessary steps to produce the final product leather. Leather offcuts are low water content waste resulting from the trimming or cutting of a large piece of leather for upholstery. Generally, this waste is generated in the automotive industry, in furniture production, or footwear industry. Besides collagen and chromium, this waste contains numerous of other chemicals. Retanning agents (basified trivalent chromium, vegetable tannage, and/or other tannages), dyes, fat, and pigments.

2.1.1 Collagen structure and stability

It is important to understand the structure of collagen in order to understand chromium leather waste stability. Collagen is among the most common fibrous proteins and it is present in tendons, ligaments, bones, dentin, skin, arteries, cartilage, and in most of the extracellular matrix in general (Fratzl, 2008). There are at least 27 known different types of collagen in vertebrates and invertebrates, type I collagen being the most common of them in skin, tendon and bone (Birk und Bruckner, 2005). These molecules are assembled in different fibrous structures with quite different properties, such as elastic skin, soft cartilage, and stiff bone and tendon (Fratzl, 2008).

What different types of collagen molecules have in common is their occurrence in the extracellular matrix, their hydroxyproline content (amino acid specific to collagen), and that they are composed of three polypeptide chains, which form a triple helix arrangement in the core of the structure (Reich, 2007). Each of the chains, called α chain, are characterized by the repeating amino acid motif (Gly-X-Y), where glycine must always be located in the third position, and X and Y can be any amino acid (Hulmes, 2008). The combination of α chains defines the type of collagen. For type I collagen, this motif is repeated approximately 350 times, X is frequently proline, and hydroxyproline is always located in position Y (Reich, 2007).

This typical sequence leads to a triple helical structure of the collagen molecule. Hydrogen bonds and natural cross-links stabilize this arrangement resulting in high resistance to heat and enzymatic degradation, and low solubility in aqueous buffers (Meyer, 2019).

Several collagen molecules are kept together by the development of molecular cross-links between them forming the fibrils and, subsequently, different kinds of tissues. In particular, the fibrils found in the skin are rich in type I collagen but also contain a significant amount of collagen type III, typically about 20% (Wess, 2008). The stability of the material is therefore increased motivating its use in the leather industry.

2.1.2 Chromium-based tanning process

The leather-making industry needs to further stabilize collagen fibres by chemical cross-linking despite their natural collagen resistance to heat and enzymatic degradation. To transform hides into leather, the dermis has to be separated from the two other layers of the hide, the epidermis (along with the hair) and the hypodermis. Subsequently, hides are washed to complete the separation of non-collagenous components and the structure of the material is opened up in preparation for the tanning step, in which tanning agents need to penetrate the structure (Reich, 2007). The tanning agent (usually basic chromium sulfate) reacts with the collagen matrix, chromium complexes form complex bonds with the carboxyl groups in the structure. The process stabilizes the collagen matrix resulting in leather (Dhayalan et al., 2007).

These cross-links formed with the tanning agents first of all have to prevent the collagen structure from collapsing during drying. They separate the molecules which would otherwise glue together resulting in stiff parchment (Reich, 2007). Furthermore, cross-linking also enhances mechanical strength in wet state, increases the denaturation temperature, reduces the swelling capacity, and lowers susceptibility to enzymatic degradation (Avery and Bailey, 2008). Thermal stability is closely related to the intrafibrillar water content of the collagen, simply because the cross-links, natural and chemical, reduce separation of the molecules leading to dehydration of the fibres (Miles et al., 2005). The chemical cross-linking process applied to hides is known as the core of the leather manufacturing process.

The stabilization process has an impact on the denaturation temperature, the more stable the material the higher the needed temperature to break down the structure into gelatin or denatured collagen (Figure 2.1). Denaturation occurs when this material is exposed to a temperature higher than the denaturation temperature and triple helices are unlocked. This random structure loses the former high stability and can be easily degraded (Figure 2.1a to 2.1d). For soluble collagen molecules, which consist of single triple helices, the structure disintegrates into a gelatin with broad molecular weight distributions, and for tissue the structure shrinks (Meyer, 2019).

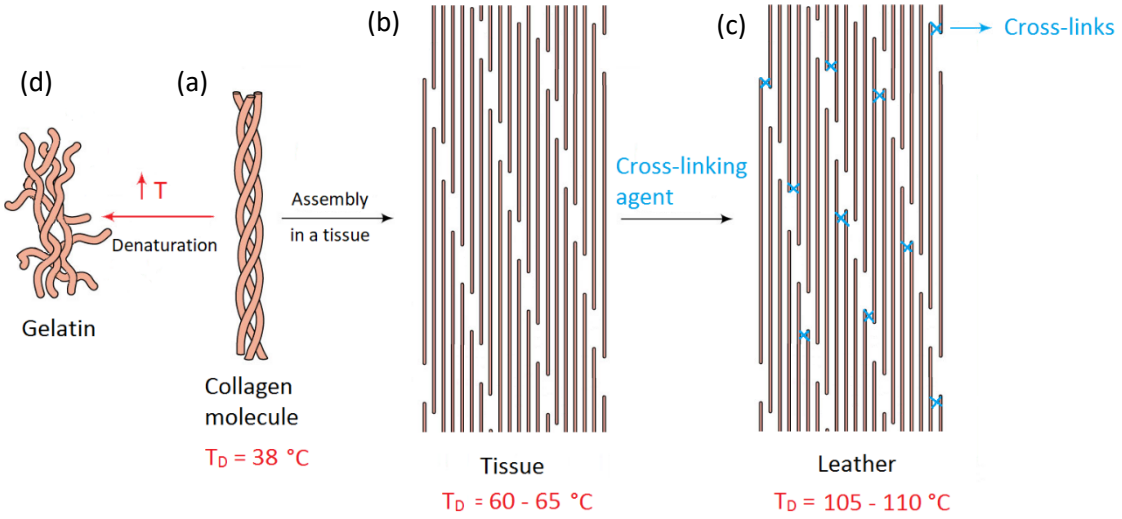

Fig. 2.1: *Different structural levels of collagen materials and their denaturation temperature (T_D). Triple helix of the collagen molecule (a), its assembly in a tissue (b), chemical cross-links formation while tanning with chromium salts (c), and collagen denaturation to gelatin through temperature exposure (d).*

The denaturation temperature of fully hydrated skin collagen is known to be 60 °C - 65 °C (Figure 2.1b), and is increased through vegetable and aldehyde tanning by up to 20 °C and through chrome tanning by up to 50 °C (Reich, 2007). Therefore, the denaturation temperature of chromium leather waste reaches 105 °C to 110 °C (Figure 2.1c), which requires a denaturation process at these temperatures (Schroepfer and Meyer, 2017).

2.1.3 Alternative management of chromium leather waste

It is possible to separate chromium leather waste into collagen protein and chromium by specific treatment. The majority of the published data on recovery of this waste concerns the alkaline hydrolysis of leather. In some cases, this process can even lead to a zero discharge method. However, the procedure is complex, time-consuming and expensive (Katsifas et al., 2004).

The literature suggests that chromium leather waste can be managed through hydrolysis of the material with alkalis, namely, CaO, $Ca(OH)_2$, MgO, or $Mg(OH)_2$, or NaOH and temperature- and/or pressure-assisted. Holloway

(1978) and Mu et al. (2003) used chromium shavings or scraps and Ferreira et al. (2014) used finished leather waste in their studies. Two products are obtained in this kind of process: protein hydrolysate, which can be used as a leather finishing agent or for animal feeding, and a chromium solution useful in tannery operations. However, the surface grain of leather treated with those chromium solutions may show a lower quality limiting its later use.

Microbial hydrolysis of chromium shavings is another option for minimum generation of waste and recovery of chromium. Pillai and Archana (2012) studied the use of *Bacillus subtilis* P13 and Katsifas et al. (2004) an *Aspergillus carbonarius* isolate. Both authors agreed that the previous autoclaving of the waste was essential for its degradation. Katsifas et al. (2004) were able to recover up to 12% of chromium, the proteinaceous solution might be used for fertilizer, feed additive or silage production. Pillai and Archana (2012) achieved a recovery of chromium of up to 50%, which could be used in tannery procedures or sold to chemical suppliers. Additionally, a valuable by-product was generated, a dehairing protease useful in the pre-tanning steps. Although those methods do not have large implementation costs, they are not very feasible since they can be regarded as very time-consuming.

Chromium leather waste can also be hydrolyzed directly by enzymes in acidic or alkaline conditions. Crispim and Mota (2003) hydrolyzed chromium leather shavings with proteases in acid and alkaline media to prepare leather board and a protein hydrolysate for leather finishing. The acidic protease (pepsin) was used to hydrolyze the chromium shavings. The resulting solution was filtered and the filtration cake was cross-linked with glutaraldehyde to prepare the waste for leather board production with no significant chromium release. The alkali protease (Rodazym ML) hydrolyzed the chromium shavings into a smaller material and then separated the chromium from the protein hydrolysate. This protein hydrolysate is able to replace all or part of the casein formulations currently applied in leather finishing. Unfortunately, the authors do not present an economic study to prove the feasibility of these processes as there is a substantial input of chemicals and energy demand.

Ferreira et al. (2010) leached finished chromium leather waste using sulfuric acid. The chromium from the obtained acid chromium extracts might be precipitated as chromium hydroxide according to Almeida and Boaventura (1997). The de-chromed material is three times more biodegradable than the original waste. However, it still contains chromium and is not acceptable at hazardous landfills since the total chromium released in the leaching test exceeds the threshold value of 5 mg L^{-1}. This treatment is nearly static and constitutes a low cost and feasible alternative but nevertheless it needs three to six days to be complete, a long time on industrial scale.

Cot et al. (2003) developed an oxidative process to recover chromium from chromium leather waste in different processing stages, whereby Cr^{3+} is oxidized to Cr^{6+} using peroxides in an alkaline medium. In this case, the treated de-chromed collagen residue can be used as substitute of casein or as pre-tanning or re-tanning resin and the recovered Cr^{3+} tanning agent resulting in extremely soft, clear grain leather. However, this method has the disadvantage that Cr^{6+} is involved, which requires an additional reductive step.

Regular adsorbents for wastewater treatment are expensive for the industry causing a growing demand of alternative adsorbents. Chromium leather shavings can be considered an alternative adsorbent and be used with or without any pre-treatment. Most authors concentrate their efforts on the adsorption of surfactants or dyes. Regarding the adsorption of surfactants, Zhang et al. (2006) tested three different kinds – anionic, cationic, and non-ionic. Whereas adsorption of the cationic and non-ionic surfactants was limited, the chromium shavings showed a high adsorption capacity of anionic surfactants. The cationic surface of chromium leather shavings attracts negatively charged molecules enhancing the probability of adsorption to occur. For the same reason, most of the papers involving dyes study the adsorption of anionic dyes (Zhang and Shi, 2004; Piccin et al., 2012 and 2013; Gomes et al., 2015). Oliveira et al. (2007) extracted chromium in advance and tested the pre-treated and the untreated leather for comparison. Results suggest that pre-treatment diminishes the effectiveness of the adsorption process. The disadvantage of this method is that there is no decrease in the amounts of this waste, much less in its toxicity. Contact with the waste may also result in the contamination of wastewater with Cr^{3+}.

As an alternative, chromium leather shavings can be used as fertilizer additive, in order to completely eliminate the waste. This is possible with (Lima et al., 2010) or without (Daudt et al., 2007) a prior treatment to remove chromium, whereas the former option entails high chemical treatment costs, and the latter will lead to soil contamination, which is restricted in many countries such as Germany, Brazil and the USA. Another way to achieve the zero solid waste target is to utilize chromium leather shavings as a protein source for poultry feed (Paul et al., 2013). For this purpose, waste underwent treatment, in order to remove chromium followed by a thermal and enzymatic treatment to produce gelatin solution. The product resulting thereof achieved the nutrient requirements for poultry feed. Some companies produce leather boards from chromium leather shavings in several countries but not all shavings satisfy the strict quality requirements for the process and therefore the elimination of waste is very restricted (IULTCS, 2018a).

All these methods represented a serious disadvantage regarding cost, time, or reutilization of the obtained products. Consequently, a more feasible method has to be investigated.

2.2 Anaerobic digestion

The anaerobic digestion is a series of microbiological processes intended to break down organic matter in the absence of oxygen. While organic matter is degraded, and therefore reduced, biogas is generated as the main product of these reactions. Biogas in turn can be used to generate energy or heat.

According to Deublein and Steinhauser (2008), benefits of the installation of a biogas plant include using biomass, which would normally be left to natural deterioration, to generate high-energy compounds and valuable fertilizers. The organic waste is also reduced to 4% after squeezing resulting in a substantial decrease of landfill area and disposal costs. Finally, biogas plants contribute to a yield increase with regard to agricultural activities on a large and small scale as biogas production is relatively simple to put into operation and subsidized

in many countries. Furthermore, the release of methane from organic waste degradation is avoided by controlled anaerobic digestion resulting in a significant reduction of greenhouse gas emissions (Marin et al., 2010).

Mata-Alvarez et al. (2014) examined the papers about anaerobic digestion published between 2010 and 2013 and concluded that the most frequent main substrates studied are animal manures (54%), sewage sludge (22%) and the organic fraction of municipal solid waste (11%). At the same time, the most commonly used co-substrates are industrial waste (41%), agricultural waste (23%) and municipal waste (20%).

2.2.1 Anaerobic digestion of tannery waste

Most biogas production papers analyzing the digestion of tannery waste focus their efforts on the digestion of untanned fleshings, sludge from wastewater treatment, wastewater, or the co-digestion of fleshings along with sludge or wastewater.

Shanmugam and Horan (2009) studied the anaerobic digestion of limed fleshings but a co-digestion with municipal solid waste was necessary to enhance the biogas yield due to the low C/N (Carbon/Nitrogen) ratio and alkaline pH. Kameswari et al. (2014a) tested different pre-treatments (ozonation, alkaline thermal treatment, and sonication) to degrade sludge from wastewater treatment and accelerate the rate of hydrolysis during the anaerobic digestion. They found that ozonation showed a higher increase in soluble chemical oxygen demand (COD) in the sludge. Banu and Kaliappan (2007) carried out a successful experiment using a hybrid upflow anaerobic sludge blanket reactor to treat vegetable tannery wastewater and achieving a COD removal of 88%. Thangamani et al. (2010) studied the co-digestion of fleshings and primary sludge from a tannery wastewater treatment plant in batch experiments and concluded that both contain a significant quantity of organic matter amenable to biodegradation. Thangamani et al. (2015) verified that the co-digestion of fleshings and effluent treatment of liquid waste from a tannery was important to minimize ammonia toxicity in a two-phase digester. Kameswari et al. (2011) studied the co-

digestion of fleshings along with pre-treated primary and secondary sludge (ozonation and ultrasonication). They were able to enhance biogas production by 53% using sludge pre-treated by ultrasonication. Kameswari et al. (2012) studied the substrate to inoculum ratio (S/I) in the co-digestion of fleshings along with mixtures of primary and secondary sludge from treatment of tannery wastewater. They concluded that a decrease of the S/I ratio beyond 1.0 has no significant influence on biogas generation and leads to an increase of the digester volume. Kameswari et al. (2014b) optimized proportions of fleshings, primary sludge, and secondary sludge as substrates to be used for co-digestion, which played a significant role in this process. A higher proportion of primary sludge enhanced the biogas production. Kameswari et al. (2015) studied the co-digestion of fleshings with primary and secondary sludge in semi-continuous mode reactors to evaluate the effect of multiple feeds on the co-digestion process. The organic load with the highest biogas production also reached the largest percentage reduction for volatile fatty acids. Only a small concentration within the range of 1.6 to 2 g L^{-1} can be found in the primary sludge. Ravindranath et al. (2015) studied the co-digestion of fleshings and tannery effluent together in an upflow anaerobic sludge blanket reactor and showed that it is possible to obtain an additional methane yield of 37.5% when using co-digestion instead of digesting the wastewater alone.

2.2.1.1 Anaerobic digestion of chromium leather waste

Dhayalan et al. (2007) and Ferreira et al. (2010) studied the anaerobic digestion of chromium leather waste. The former concluded that degradation of this waste is possible using anaerobic sludge, which however is a very slow process and leads to low biogas amounts. The latter found that the results are dependent on the anaerobic sludge concentration and origin. Agustini et al. (2015) also studied the degradation of chromium leather shavings and detected 55% of methane in the produced biogas. Priebe et al. (2016) found a low performance of the chromium shavings to generate biogas and associated this with the saturation of the reactive sites due to chromium bonding and low water solubility. More recently, Agustini et al. (2018) concluded that a scale-up of the anaerobic digestion of chromium shavings (300 mL to 2.5 L digesters) can increase biogas yields and destruction of organic matter. In all cases, the experiments were

batch tests which lasted one to four months, and in most of the studies more than ten days were needed to start the biogas production. For instance, Agustini et al. (2015), in studies concerning the anaerobic digestion of chromium shavings, needed more than twenty days to start a significant production of biogas.

2.2.1.2 Destabilization of chromium leather waste

As previously mentioned, the tanning process increases the stability of collagen transforming it into leather which is resistant to enzymatic degradation. It is well known that enzymatic attack and decay of leather in soil is still possible, but only very slowly (Reich, 2007).

Anaerobic digestion is based on enzymatic degradation. Denaturation of the leather structure is a possibility to ease the enzymatic attack and enable the process. The prior denaturation of the structure can be accomplished increasing the temperature to the denaturation temperature or higher (105 °C – 110 °C).

Most of the research concerning the pre-treatment of substrates for biogas production addressed mechanical pre-treatments (33%), such as ultrasound and extrusion, followed by thermal pre-treatments (24%), such as steam explosion and autoclave and chemical pre-treatments (21%) (Mata-Alvarez et al., 2014). Mechanical pre-treatments are able to reduce particle size and, consequently, increase the specific surface available to the medium, which could improve gas production and lead to more rapid digestion (Mata-Alvarez et al., 2000). Furthermore, pre-treatments help to breakdown complex polymers into smaller molecules promoting hydrolysis (Penaud et al., 1999), the stage responsible for limiting the rate of degradation. The main goal is to render a more biodegradable substrate to the digestion process thus increasing methane production (Mata-Alvarez et al., 2014).

Some studies were published on the pre-treatment of chromium leather waste. As mentioned before, Pillai and Archana (2012) and Katsifas et al. (2004) concluded that the previous autoclaving of chromium leather shavings was essential for its degradation by microorganisms. Therefore, a pre-treatment prior to

anaerobic digestion can denature the chromium leather waste and start hydrolysis of the material in order to ease anaerobic digestion and improve the biogas production.

2.2.2 Microbiology: trophic groups of the anaerobic digestion

The production of biogas is accomplished through anaerobic digestion of organic matter. This is a quite complex microbial process that occurs in the absence of oxygen with many types of strict and facultative anaerobic bacteria (Murphy and Thamsiriroj, 2013; Deublein and Steinhauser, 2008). This process can be divided into four phases – hydrolysis, fermentation or acidogenesis, acetogenesis, and methanogenesis, according to the decomposition process of the substrates and the bacteria acting to degrade them (Figure 2.2).

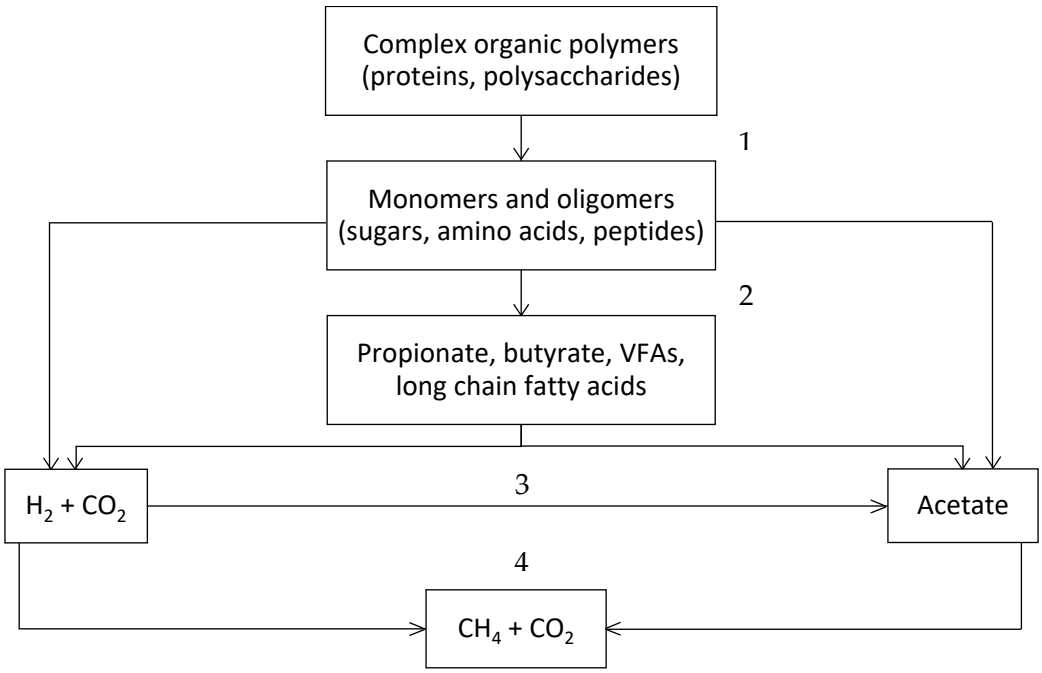

Fig. 2.2: *Phases of anaerobic digestion (1) hydrolysis, (2) fermentation or acidogenesis, (3) acetogenesis, and (4) methanogenesis (adapted from Murphy and Thamsiriroj, 2013).*

1 - Hydrolysis: Exoenzymes (hydrolase) of hydrolytic bacteria break down polymers (undissolved compounds) into monomers (water-soluble fragments), the covalent bonds are split in a chemical reaction with water (Figure 2.3).

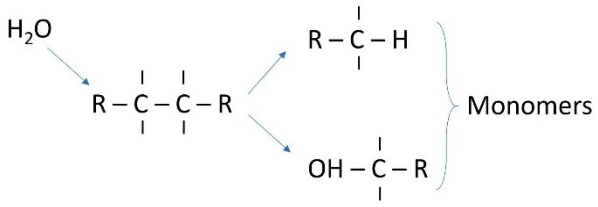

Fig. 2.3: *Formation of monomers (adapted from Deublein and Steinhauser, 2008).*

If the substrate is highly complex and therefore difficult to degrade, the hydrolytic stage limits the rate of degradation. The hydrolysis time varies with the substrate, in the case of proteins, such as collagen, it is a matter of days (Deublein and Steinhauser, 2008).

2 - Fermentation or acidogenesis: The fermentative bacteria ferment the resultant monomers from the hydrolysis into acetic acid, hydrogen, carbon dioxide and volatile fatty acids such as propionate, butyrate and alcohols. The concentration of the hydrogen formed intermediately affects the kind of products developing in this phase. The higher the partial pressure of hydrogen, the fewer acetate is formed (Deublein and Steinhauser, 2008).

Amino acids, products of the hydrolysis of proteins, are mainly degraded through the Stickland Reaction (Schink and Stams, 2013; Ramsay and Pullammanappallil, 2001). This reaction is based on the coupled deamination between two amino acids acting as donor and acceptor of hydrogen. The products of digestion are ammonia, carbon dioxide, and volatile fatty acids, which vary from the coupled amino acids being digested and the present bacteria in the medium (Nisman, 1954). Equation 2.1 shows the example of the coupled deamination of alanine and glycine.

$$CH_3CHNH_2COOH + 2NH_2CH_2COOH + 2H_20 \rightarrow 3CH_3COOH + 3NH_3 + CO_2 \quad (2.1)$$

Alanine Glycine Acetic Acid

The main proteolytic bacteria in sludge are gram-positive bacteria, principally Clostridia. The majority of these bacteria are known to degrade amino acids through the Stickland Reaction (McInerney, 1988). This reaction is the main way

these bacteria obtain energy to grow when amino acids are the only source of carbon and nitrogen (Nisman, 1954).

3 - Acetogenesis: Acetogenic bacteria convert the fermentative intermediates (volatile fatty acids) into methanogenic substrates, hydrogen, carbon dioxide, acetic acids and unicarbon compounds. Even though these bacteria are obligatory H_2 producers, this product is toxic to them. They must act in symbiosis with bacteria in a different trophic group (methanogenic organisms) as they can only survive and grow if the hydrogen partial pressure is kept at a very low level. If the hydrogen partial pressure is low, the acetogenic bacteria will mainly produce H_2, CO_2, and acetate, otherwise the main products will be butyric, capronic, propionic, and valeric acids and ethanol. From these products, only the three named first can be processed by methanogenic archaea.

The homoacetogenic bacteria reduce H_2 and CO_2 to acetic acid (Equation 2.2). Although these bacteria (hydrogen-consuming acetogens) are not able to compete with methanogens for hydrogen, they help to maintain low hydrogen partial pressures and increase the amount of acetate available for the methanogenesis (Deublein and Steinhauser, 2008).

$$2CO_2 + 4H_2 \leftrightarrow CH_3COOH + 2H_2O \tag{2.2}$$

4 - Methanogenesis: Methane is generated in two different ways under strictly anaerobic conditions. About 30% of the whole methane is produced by hydrogenotrophic methanogenic archaea. In symbiosis with the acetogenic bacteria, they utilize the H_2 produced in the previous step and ensure very low hydrogen partial pressure through the reduction of CO_2 by H_2:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \tag{2.3}$$

Aceticlastic methanogenic archaea are responsible for about 70% of methane produced using acetic acid as substrate (Equation 2.4). Unlike the hydrogenotrophic methanogens these archaea are relatively inefficient in acetate uptake and have slow reproduction rates (Deublein and Steinhauser, 2008).

$$CH_3COOH \leftrightarrow CH_4 + CO_2 \tag{2.4}$$

2.2.3 Biogas production studies

Biogas production trials can be carried out discontinuously or continuously. They should be performed in a gastight reactor to assure the absence of oxygen. A substrate, composed mostly of organic matter, and an inoculum, preferable a sludge with a diversified biocoenosis, are placed inside the reactor. After sufficient time of contact, biogas is produced as a result of the interaction between substrate and inoculum.

2.2.3.1 Batch trials

Laboratory batch trials are essential to investigate biogas production. It allows multiple experiments to be run simultaneously testing numerous variables and collecting large amounts of data in relatively short periods of time. The bench-scale tests reduce the amount of materials required for the trials thus minimizing waste generation and costs. This type of research helps to determine feasibility of the process, and to optimize it in order to improve waste treatment and biogas production (Gamble et al., 2015). These trials enable evaluation of the biogas yield and the biogas degradability of a material, the speed of anaerobic degradation, and its inhibitory effect within the range of tested concentrations (VDI 4630, 2006).

In batch tests, the reactor is filled all at once. The substrate to inoculum mixture remains in the closed reactor until the end of the pre-specified digestion time. At the end of digestion, the resulting biomass is removed for further analyses (Gamble et al., 2015).

2.2.3.2 Continuous trials

Continuous tests simulate long-term process conditions, which allow to investigate capabilities and loading limits of the process, mean residence time, and formation and accumulation of metabolic intermediates and their influence on process stability. The apparatus is larger and necessary supervision higher for continuous trials than for batch tests and several measurements of a large number of parameters in the gas and liquid phases (VDI 4630, 2006) are required.

In continuous tests, the substrate to inoculum mixture is added to the digester at pre-designated times. Most large-scale industrial digesters operate in continuous mode as it allows the digester to continually produce biogas (Gamble et al., 2015).

2.2.4 Inhibitors

2.2.4.1 Chromium content

Heavy metals such as chromium are essential for bacterial growth in very small quantities, but higher quantities have a toxic effect (Abbasi et al., 2012). In particular, lead, cadmium, copper, zinc, nickel, and chromium can lead to disturbances in biogas plants (Deublein and Steinhauser, 2008).

Chromium acts as trace element at low concentrations stimulating the activity of the bacteria. The minimum amount of trace element required is 0.005 – 50 mg L^{-1}. The toxic effect starts at concentrations higher than 28 - 300 mg L^{-1} as free ions or 530 mg L^{-1} as carbonate (Deublein and Steinhauser, 2008). Gayatri et al. (2000) were able to demonstrate that the deactivation of collagenase by unbound chromium complexes contained in chromium-tanned leather is possible. The direct binding of chromium complexes to the enzyme was proved. Leather contains up to 1500 mg kg^{-1} of non-bonded Cr^{3+} complexes and it must be assumed that other enzymes are also susceptible to this effect (Reich, 2007). Consequently, anaerobic digestion can be inhibited.

2.2.4.2 Hydrogen sulfide (H_2S)

The sulfate-degrading bacteria act to reduce sulfate forming hydrogen sulfide following Equation 2.5. This is problematic for the methane formation because these bacteria compete with the hydrogenotrophic methanogenic archaea for hydrogen, and hydrogen sulfide is toxic to methanogenics (Polster and Brummack, 2005). As sulfate-degrading bacteria need less energy and do not need a symbiosis partner, this process disturbs the methane formation and causes overacidification.

$$4H_2 + 2H^+ + SO_4^{2-} \rightarrow H_2S + 4H_2O \qquad (2.5)$$

Hydrogen sulfide escapes with the biogas and is dissolved in undissociated and dissociated form in the substrate as weak acid, developing hydrogen sulfide ions (HS^-) and sulfide ions (S^{2-}). With decreasing pH, the dissolved undissociated hydrogen sulfide concentration rises and works as a cellular poison at concentrations higher than 50 mg L^{-1}. Hydrogen sulfide can also cause inhibition due to precipitation of essential trace metals as insoluble sulfides (Deublein and Steinhauser, 2008).

2.2.4.3 Volatile fatty acids

The most commonly formed volatile fatty acids in biogas reactors are acetic acid, propionic acid, and isobutyric acid. The sum of the concentration of all volatile fatty acids formed should be lower than 4 g L^{-1} to prevent inhibition. The concentration of acetic acid alone should be lower than 3 g L^{-1}, that of propionic acid lower than 1 g L^{-1}, and that of isobutyric acid lower than 0.5 g L^{-1} (Kaiser et al., 2008). However, a propionic acid concentration of 0.3 g L^{-1} only is sufficient to disturb anaerobic digestion (Deublein and Steinhauser, 2008).

The production of volatile fatty acids leads to a decrease of the pH value, even though they are intermediate products for generating biogas. The growth of methanogenic archaea is inhibited if the pH value is below 6.5, but the acidogenic bacteria continue to work until the pH value drops to 4.5. Consequently, there is a fast accumulation of volatile fatty acids (Murphy and Thamsiriroj, 2013). The drop in pH value is buffered by formation of alkalinity through CO_2 production. For this reason, using the pH value as an indicator for the stability of anaerobic digesters is not reliable (Kaiser et al., 2008). The volatile fatty acids concentration should be controlled.

2.2.4.4 Inhibition by diauxie

The biogas formation curves in batch trials are defined as the difference between the biogas production of the substrate less the biogas production of the inoculum. They can also indicate if digestion works adequately. This qualitative information enables the detection of inhibition in the reactor and that of difficult substrates. Figure 2.4 shows the typical shapes of biogas formation curves.

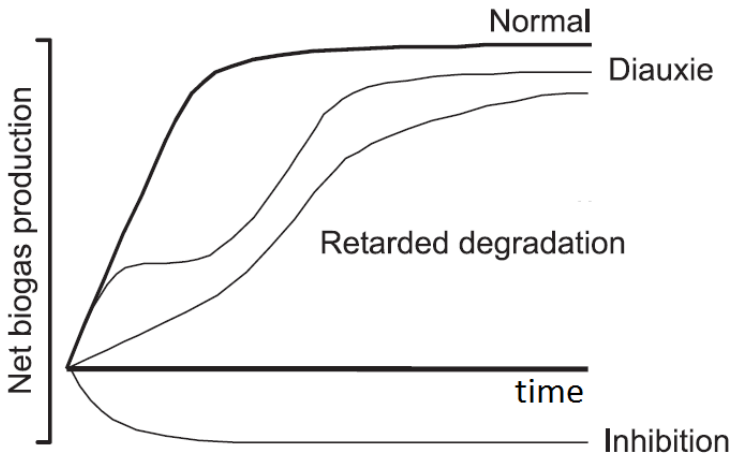

Fig. 2.4: *Typical shapes of biogas formation curves (adapted from VDI 4630, 2006).*

If the substrates to be digested are easily convertible substances, they are converted rapidly into biogas and the characteristic curve shows a steep increase in the accumulated biogas quantity. For complex substrates, which are difficult to degrade, biogas production is delayed and a retarded gas formation curve is typical for that. A retarded gas formation curve can also be caused by slight inhibition. If biogas production is strongly or completely inhibited, curves indicate a negative net biogas production – in other words, gas formation is less than that of the batch from the zero sample. In addition to the curve shapes shown here, there is a large number of mixed forms (VDI 4630, 2006).

If degradation occurs in two stages (the curve resembles stairway steps), this indicates a two-phase decomposition, also known as diauxie (VDI 4630, 2006). The diauxie curve is characterized by a plateau-phase in the middle of the biogas production. After that the system recovers, and substrates continue to be transformed into biogas. This inhibition increases the time necessary for the complete digestion of the substrate, which has a negative effect on the biogas production.

2.2.5 Theoretical biogas yield: Buswell equation

The maximum biogas yield expected can be calculated using the Buswell equation after Boyle (1976) (Equation 2.6) if the basic elementary formula of the substrate is known.

$$\begin{aligned} C_aH_bO_cN_dS_e + \left(a - \frac{b}{4} - \frac{c}{2} + \frac{3d}{4} + \frac{e}{2}\right) H_2O \rightarrow \\ \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{8} - \frac{e}{4}\right) CH_4 + \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4} + \frac{3d}{8} + \frac{e}{4}\right) CO_2 \\ + dNH_3 + eH_2S \end{aligned} \quad (2.6)$$

It is important to bear in mind that this is a theoretical value, which will probably not be achieved because part of the substrate, 3 to 10%, is converted into biomass and is not available for biogas formation. For proteins, a conversion rate of 50 to 70% is expected (VDI 4630, 2006).

2.3 The use of chromium leather waste in the biogas production

Hitherto the use of chromium leather waste to produce biogas has not been studied in depth. Some studies proved that degradation of this waste through anaerobic digestion is possible but very slow and leads to low biogas amounts. The substrate is challenging due to the natural structure of collagen and chemical cross-links acquired in the tanning process, which provide high stability to anaerobic digestion. Using pre-treatments is a possibility to ease the hydrolysis step in the anaerobic digestion and improve biogas production.

The present work will illustrate the use of different pre-treatments with regard to chromium leather waste. This approach to produce biogas has not been studied to date. Different mechanical and thermal pre-treatment methods are considered and their degradation results are presented.

Investigation of the biogas production using untreated and pre-treated chromium leather waste as substrate was performed in batch and continuous reactors. Whereas some studies exist for the treatment of chromium leather waste under batch conditions, there is no paper published on continuous reactors for this material.

Aim of the present investigation was to:

1. Characterize the pre-treated chromium leather waste to predict their performance in the anaerobic digestion.
2. Prove the advantages of using pre-treated chromium leather waste instead of the untreated substrate to produce biogas in batch and continuous reactors.
3. Determine the causes of inhibition in the batch and continuous reactors.

3 Materials and Methods

Three different steps were carried out in this study. At first, chromium leather waste was pre-treated to initiate material degradation. Three common thermal and/or mechanical techniques are considered for this purpose – extrusion, hydrothermal treatment, and autoclave. Secondly, the pre-treated material was assessed regarding its degree of degradation using different fast in vitro methods, e.g. enzymatic degradability and calorimetric analyses. Finally, the biogas production of the waste was investigated through biogas production trials to prove the feasibility of using chromium leather waste to produce biogas.

3.1 Materials

The collagen-based materials used as substrate to produce biogas were chromium leather waste. Chromium shavings (shaved from wet chromium-tanned leather) and automotive leather offcuts (trimmed from finished leather ready for use in car manufacture) were tested for this purpose (Figure 3.1). The materials were obtained from a local tannery (HEWA Leder, Freiberg, Saxony, Germany).

They were characterized regarding their water content (DIN EN ISO 4684, 2005), inorganic matter (DIN EN ISO 4047, 1998), and total chromium as chromic oxide content (DIN EN ISO 5398-1, 2007) in per cent by mass. Experiments were run in triplicate. The chromium leather waste tested is characterized in Table 3.1. Usually, processed chromium shavings show water contents of 40 to 50% but those used in this work had already been air-dried to some extent and have a water content of almost 20%.

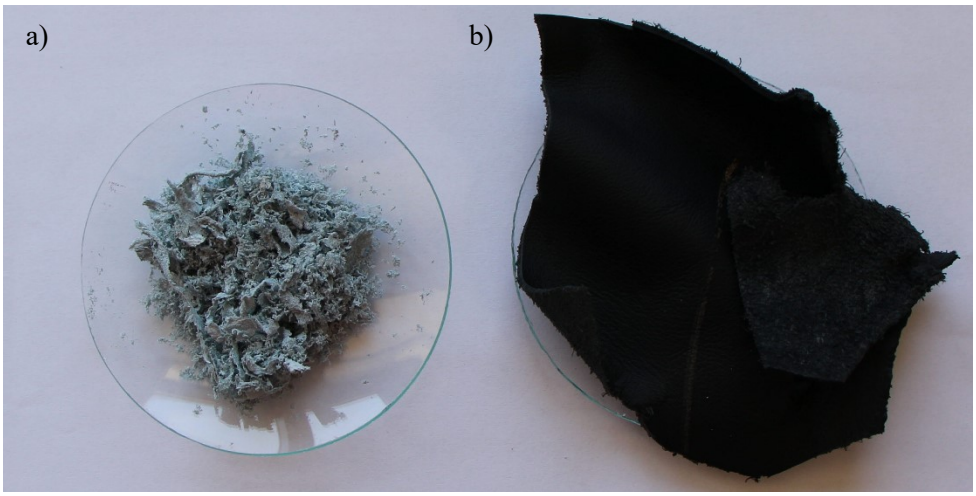

Fig. 3.1: *Chromium shavings (a) and leather offcuts (b).*

Tab. 3.1: *Chromium shavings and leather offcuts characterization.*

	Chromium shavings (CS)	**Offcuts**
Water content (%)*	19.7 ± 0.1	11.4 ± 0.0
Inorganic Matter (%)**	11.2 ± 0.1	6.4 ± 0.0
Chromium (%)**	4.6 ± 0.0	4.1 ± 0.1

* Mean ± standard deviation, n = 3

**Dry basis; mean ± standard deviation, n = 3

White hide powder supplied by the research institute FILK (Freiberg, Germany) was used in chemical analyses. This material is bovine hide (pelt), which was chemically unhaired and does not contain any trivalent chromium (Schroepfer and Meyer, 2017). Bovine hide gelatin (260 g Bloom, type B) kindly supplied by Gelita AG (Eberbach, Germany) was used as a reference in the biogas trials. Gelatin is a hydrolysate of collagen with short chains. Chemically, gelatin is the same as collagen only differing in the structure. Gelatin is known to be soluble and easily degradable by enzymes.

For the biogas trials, two different inocula were used. For batch trials, the mesophilic anaerobic inoculum was anaerobic sludge from the municipal sewage treatment plant (Freiberg, Germany). A reference substrate (microcrystalline cellulose) was also fermented in every batch to ensure that the seeding sludge used has a proper biological activity. For continuous trials, mesophilic anaero-

bic sludge from the tannery SÜDLEDER (Rehau, Germany) was used. This inoculum showed some quantities of chromium and collagen content (about 1% chromium content and 3% collagen content on dry basis) because it was produced in a tannery.

3.2 Pre-treatments of the chromium leather waste

Different heat and mechanical pre-treatment techniques were tested in order to denature the chromium leather waste and promote its degradation and transformation into biogas. Extrusion, a classical technique from the polymer industry; a continuous hydrothermal treatment, which is commonly used to plastify wood for the manufacture of wood composites; and autoclave, a very commonly available instrument in laboratories, were used to pre-treat and denature the chromium shavings and leather offcuts. Whereas during extrusion the material is affected by heat, mechanical shear, and pressure, the autoclave and hydrothermal treatments are based on heat and steam pressure only. During the process, temperatures higher than the denaturation temperature were achieved in order to enable enzymatic degradation to produce biogas.

3.2.1 Extrusion

Extrusion was performed on chromium shavings and leather offcuts using a co-rotating twin-screw-extruder Werner & Pfleiderer ZSK 25 (Figure 3.2) at different temperatures and humidity conditions (dry or wet) in a continuous process. Prior to that, the wet chromium shavings were moistened with water (60.1% water content), well homogenized and left overnight. The dry chromium shavings (19.7% water content) are air-dried chromium shavings. The leather offcuts were extruded as received from the tannery (11.4% water content).

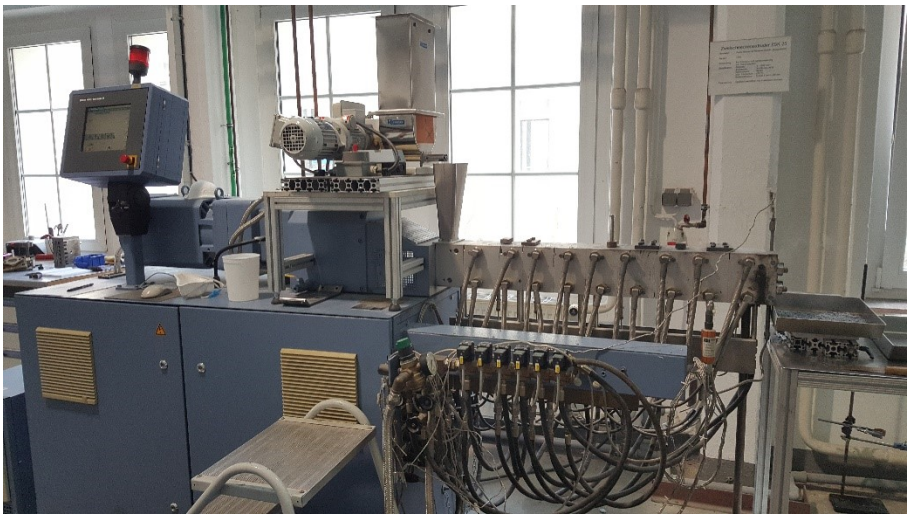

Fig. 3.2: *Extruder Werner & Pfleiderer ZSK 25.*

This extrusion process starts by feeding the sample from a hopper into the barrel of the extruder. The material is gradually degraded due to the mechanical energy generated by turning screws and by heaters arranged along the barrel. The conversion of mechanical energy into heat makes it possible to use this process even below the denaturation temperature of chromium-tanned leather (105 °C to 110 °C). The process takes approximately three minutes (Figure 3.3).

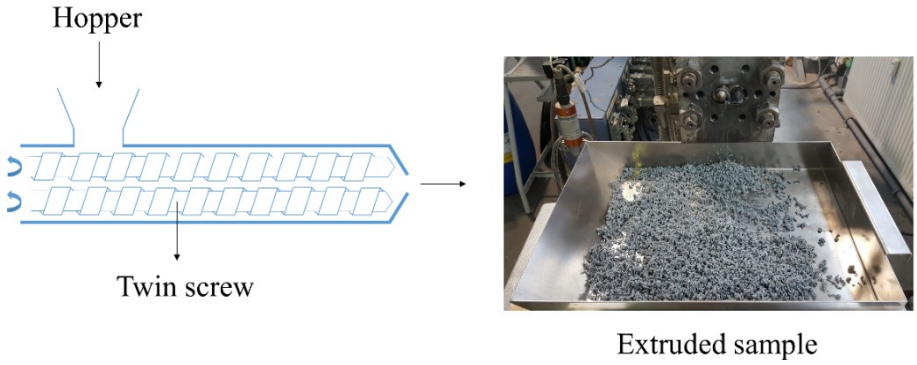

Fig. 3.3: *Operation of the extruder machine*

The pre-treated shavings that originated from dry and wet chromium shavings differ in appearance (Figure 3.4). Extrusion of dry chromium shavings resulted in a powdered sample. In contrast, the wet chromium shavings resulted in samples with granular shape. A high water content opens up the structure of the

sample increasing the space between the fibres. This eases extrusion and enables formation of small grains of leather. If leather having a low water content is extruded, the fibres are close to one another. Consequently, when the leather structure was broken using mechanical force, the pieces of leather became very small and a powder was generated. A powder with some small leather pieces can be seen for the extruded offcuts.

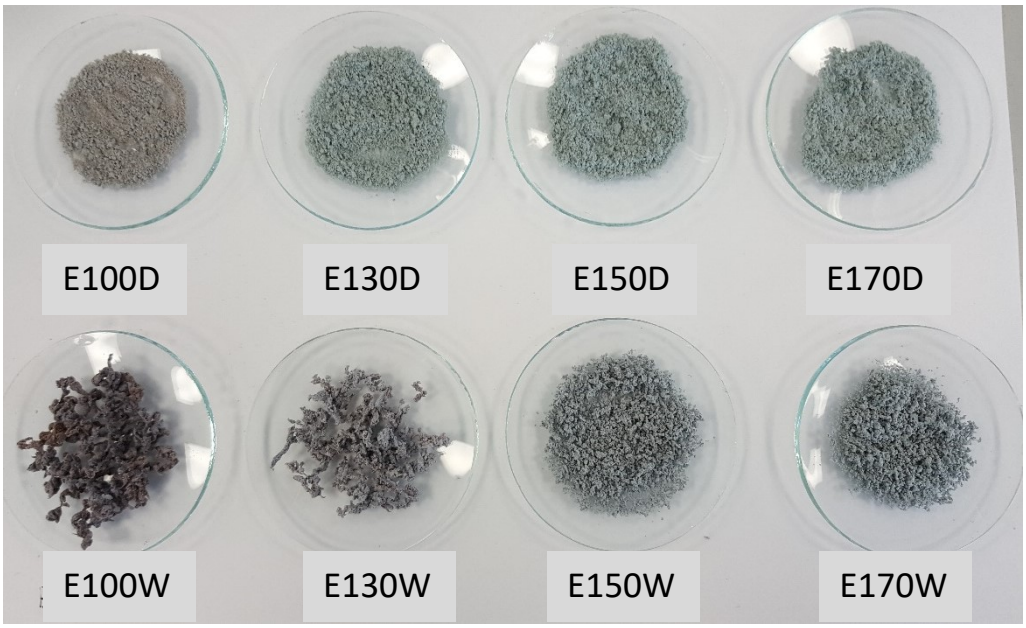

Fig. 3.4: *Chromium shavings extruded (E) dry (D) or wet (W).*

Tab. 3.2: *Extruded samples and their pre-treatment conditions.*

Sample	Pre-treatment Temperature (°C)	Pre-treatment water content (%)*
E100D	100	19.7 ± 0.13
E130D	130	
E150D	150	
E170D	170	
E100W	100	60.1 ± 0.19
E130W	130	
E150W	150	
E170W	170	
EO	170	11.4 ± 0.0

*Mean ± standard deviation, n = 3

The letter E in the nomenclature of the extruded samples stands for extrusion, the number is the extrusion temperature, and the letter D or W at the end stands for dry or wet, respectively. EO stands for extruded offcuts. Table 3.2 lists the samples nomenclature and pre-treatment conditions.

3.2.2 Hydrothermal treatment

The chromium shavings were subjected to hydrothermal treatment through a continuous autoclave system attached to a refiner (Andritz CPH 12-1) at the Institut für Holztechnologie (Dresden, Germany). Usually this equipment is used to plastify wood chips but it is also adequate to process a variety of organic materials. The process was carried out at different temperature and pressure conditions in saturated steam (Figure 3.5).

Fig. 3.5: *Continuous autoclave and refiner Andritz - front (a) and back view (b).*

The temperature was adjusted regarding the saturated steam relative pressure, however due to technical reasons the temperature was not as exact as expected. The material was dosed into a digester in which it was denatured with steam under pressure. Pre-treatment time was about 45 seconds.

The shavings treated hydrothermally were also different in appearance. Those that were pre-treated at 170 °C had the consistency of a liquid that solidified into a gel-like mass after cooling. Chromium shavings pre-treated at 150 °C and 140 °C were very similar, they appeared more like a dough after cooling. Figure 3.6 shows the shavings treated hydrothermally after cooling. The letter H in the nomenclature of the pre-treated sample stands for hydrothermal treatment and the number is the treatment temperature. Table 3.3 lists the samples nomenclature and pre-treatment conditions.

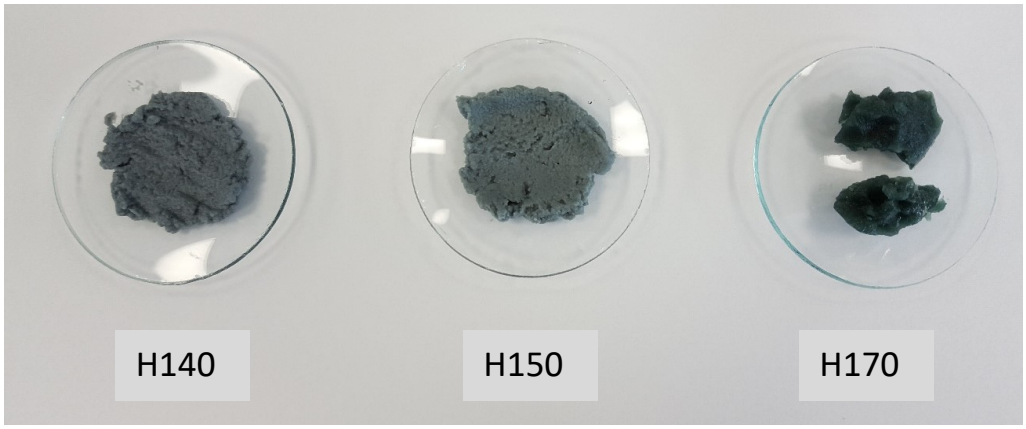

Fig. 3.6: *Chromium shavings treated hydrothermally (H) after cooling.*

Tab. 3.3: *Samples treated hydrothermally and their pre-treatment conditions.*

Sample	Pre-treatment Temperature (°C)	Pre-treatment Pressure (bar)
H140	141	2.2
H150	152	3.8
H170	169	7

3.2.3 Autoclave

Chromium shavings were autoclaved on laboratory scale. The trials were carried out in a minimized system in order to reproduce the autoclaving conditions (high temperature and pressure). Screw cap micro tubes tightly closed through O-ring sealing and a block heater (Stuart SBH130D) at 120 °C (Figure 3.7) were used. This apparatus allows for better control of the pre-treatment time and temperature, which would not be possible using a laboratory autoclave. The

laboratory autoclave would need to be sealed with the chromium shavings inside at room temperature, hence the material would be exposed to high temperatures during preheating of the autoclave.

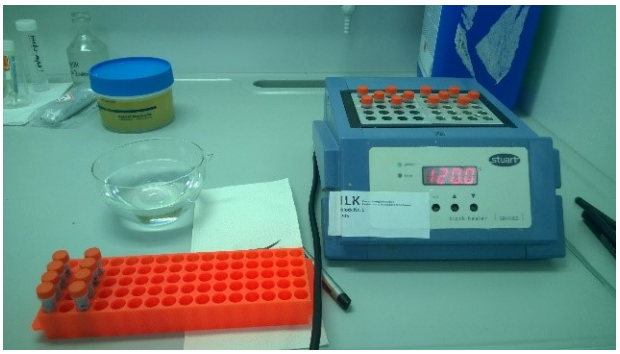

Fig. 3.7: *Laboratory equipment used as autoclave.*

Chromium shavings were moistened in advance with distilled water until the saturation point was reached, and left overnight at room temperature. The samples were placed in the micro tubes, tightly closed, and subsequently placed in the block heater at 120 °C. The micro tubes were preheated for 3 minutes and 30 seconds, the estimated time necessary for the samples to reach the autoclaving temperature (120 °C). Each sample was exposed to the autoclaving conditions for a predetermined time. Thereafter the samples were dried in a drying oven at 30 °C for one day.

After drying, the autoclaved shavings were dry and brittle, and in case of longer pre-treatment times gel-like areas began to emerge. These areas can be seen from 96 minutes of pre-treatment onwards, and their frequency of appearance increases based on the pre-treatment time (Figure 3.8). The letter A in the nomenclature of the pre-treated sample stands for autoclaved and the number is the pre-treatment time. The pre-treated samples nomenclature and pre-treatment conditions are also shown in Table 3.4.

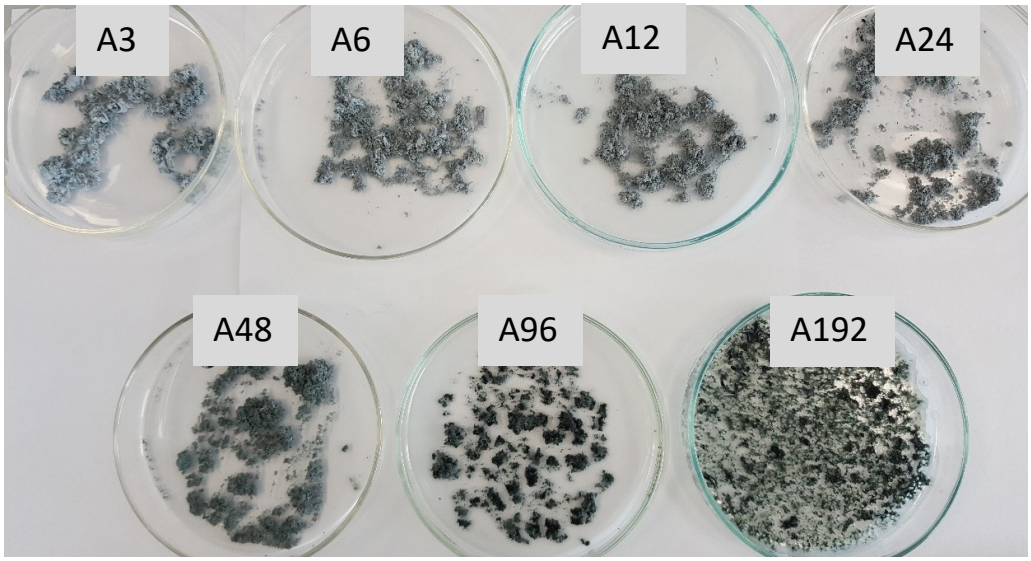

Fig. 3.8: *Autoclaved (A) chromium shavings after drying.*

Tab. 3.4: *Autoclaved samples and their pre-treatment time.*

Sample	Pre-treatment time (min)
A3	3
A6	6
A12	12
A24	24
A48	48
A96	96
A192	192
A384	384

3.3 Chemical analyses

3.3.1 Analyses of the pre-treated chromium leather waste

The pre-treated and untreated chromium leather waste was characterized and evaluated by scanning electron microscopy (SEM), differential scanning calorimetry (DSC), digestibility by trypsin and collagenase, and solubility in water.

3.3.1.1 Samples characterization

The pre-treated and untreated samples were characterized regarding their water content (DIN EN ISO 4684, 2005), inorganic matter (DIN EN ISO 4047, 1998), and chromic oxide content (DIN EN ISO 5398-1, 2007) in per cent by mass.

The collagen content of the pre-treated and untreated chromium leather waste was calculated through determination of the hydroxyproline content (Stegemann, 1958) and multiplying this value by the factor 7.46 (100 g of protein contain 13.45 g of hydroxyproline) (Reich, 1966). Samples were hydrolyzed with hydrochloric acid, oxidized with chloramine T, and then reacted with p-dimethylamino-benzaldehyde to develop a red chromophore. For gelatin, no collagen chains can be found in the structure but it is possible to detect amino acids. For this reason, only the hydroxyproline content was determined.

3.3.1.2 Scanning electron microscopy (SEM)

The surfaces and fibres of the pre-treated and untreated chromium leather waste were investigated using SEM (FEI QUANTA FEG 250). The images obtained using this method were compared visually to evaluate the effect of the different pre-treatments on chromium leather waste. Energy-dispersive X-ray spectroscopy (EDX) was used to identify the type of elements that exist in the samples in case they affect the anaerobic digestion.

3.3.1.3 Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) is a useful technique to study the thermal stability of collagen-based materials (Miles et al., 2005). Using DSC, the enthalpy of the denaturation process was evaluated and it was checked whether the pre-treated chromium leather waste has already been denatured or not. In DSC trials, the difference between the amount of heat required to increase the temperature of the studied material and a reference is measured as a function of temperature. As seen in Figure 3.9, the onset temperature is the denaturation temperature of the studied material and the area below the denaturation peak is the heat of denaturation used to calculate the enthalpy of denaturation.

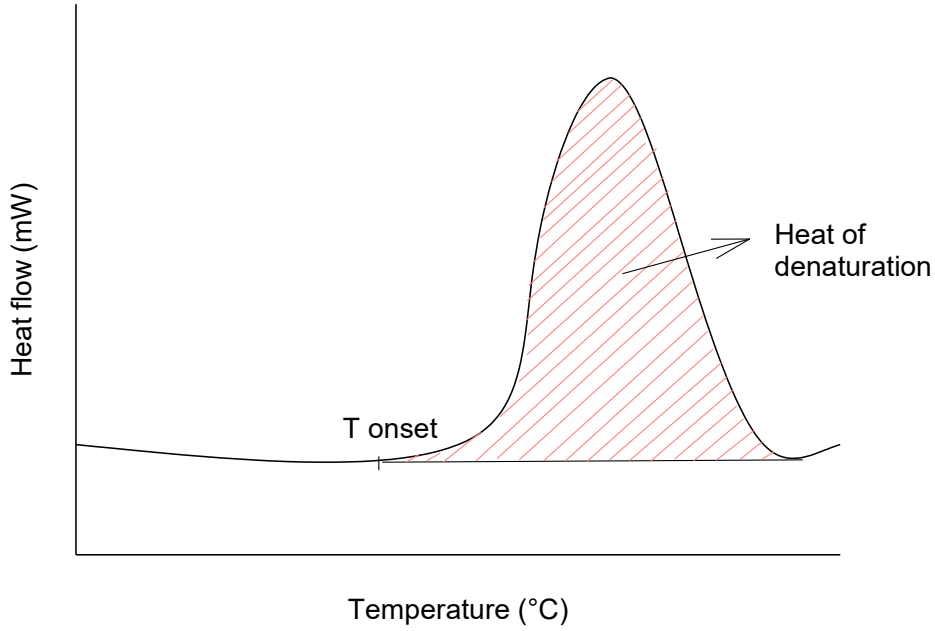

Fig. 3.9: *Example of a DSC curve.*

For collagen-based materials, the enthalpy represents the necessary energy to break down the hydrogen bonds that stabilize the triple helix (Miles and Ghelashvili, 1999). Thermal profiles in fully hydrated state of the pre-treated and untreated chromium leather waste were taken at temperatures between 0 and 130 °C using DSC (DSC 1 STAR^e System Mettler Toledo) to assess thermal changes as a function of input temperature. As the denaturation temperature of collagen varies with the pH value (Schröpfer, 2012), the pH value was previously adjusted to 7 by washing of the samples with a KH_2PO_4/K_2HPO_4 buffer solution.

3.3.1.4 Enzyme assays

Susceptibility of the pre-treated and untreated chromium leather waste to enzymatic degradation was evaluated with enzyme assays. Enzymes break down peptide bonds in the backbone of the structure, a process similar to the actual hydrolysis in the anaerobic digestion. The enzyme assays are based on the measurement of degradation of the samples. As the non-denatured collagen is stable to enzymatic degradation, it is possible to determine the fraction of the samples which was degraded during pre-treatment.

Trypsin trials were carried out using safe-lock microcentrifuge tubes (2 mL) and a block heater (Stuart SBH130D). At first, samples were placed in the microtubes with a NH_4HCO_3 buffer solution adjusted to pH 8 and left overnight in a laboratory refrigerator. Then the 0.01% trypsin solution (SIGMA, 1382 U mL^{-1}) was added at a temperature of 37 °C over a period of five hours and, finally, washed out with distilled water. Experiments were run in triplicate and a t-test was conducted to compare means.

Collagenase trials were carried out using chromium shavings and shavings extruded dry at 100 °C. Unlike other proteases, collagenases are capable of destabilizing the triple helical collagen (Meyer, 2019). Trials were carried out using safe-lock microcentrifuge tubes (2 mL) and a thermomixer (Thermomixer comfort, Eppendorf) with agitation at 750 rpm. At first, samples were placed in the microtubes with a NH_4HCO_3 buffer solution adjusted to a pH value of 7.5. A 0.05 M solution of $CaCl_2$ was added to provide Calcium ions required for enzyme stability and activity. Then the collagenase solution (SIGMA, ≥ 125 CDU mg^{-1}), in a concentration of at least 0.2 CDU per mg of substrate, was added. The trials were conducted at a temperature of 37 °C over different periods of time of up to seven days. Every two days collagenase solution was added to the microtubes. Finally, the samples were washed out with distilled water. Experiments were run in duplicate.

The degradation (D) by trypsin or collagenase is the portion of the sample that solubilizes in water after enzymatic treatment and it is represented by Equation 3.1:

$$D = 100 - \left(\frac{m_f}{m_0}.100\right)\% \qquad (3.1)$$

Where m_0 (mg) is the onset mass of the sample and m_f (mg) is the mass after the reaction time, both masses were considered on a dry basis.

3.3.1.5 Solubility in water

It is known that for identical amino acid compositions, the water solubility of a protein is correlated with the chain length and number of cross-links and can therefore be used to measure the degree of degradation (Klüver and Meyer,

2013). In this experiment the parameters expected inside of an anaerobic reactor such as temperature and pH value are reproduced in order to evaluate the portion of the sample which is promptly soluble.

The solubility of the pre-treated and untreated chromium leather waste in water was evaluated at a temperature of 37 °C. Approximately 15 mg of sample on dry basis was placed in safe-lock microcentrifuge tubes (2 mL) with 1.5 mL of water and its pH value was adjusted between 7 and 8 (pH meter Mettler Toledo) with a NH_4HCO_3 solution. The samples were stirred in a thermomixer (Eppendorf) at 300 rpm at the desired temperature for 2 hours. Afterwards the samples were centrifuged, the supernatant was discarded, the centrifugate dried, and the dry mass determined. Experiments were run in triplicate and a t-test was conducted to compare means. The solubility in water (SW) is represented by Equation 3.2:

$$SW = 100 - \left(\frac{m_f}{m_0}.100\right)\% \quad (3.2)$$

Where m_0 (mg) is the onset mass of the sample and m_f (mg) is the mass after the treatment, both masses were considered on a dry basis.

3.3.2 Analyses of the biomass

3.3.2.1 Degradation degree of collagen

Since the pre-treated and untreated chromium leather waste used as substrate in the anaerobic digestion is collagen-based material, the collagen content of the substrates and in the final biomass was measured (after anaerobic digestion) in order to calculate the degradation of the substrate during digestion.

The degradation of the collagen at the end of the trials, the degradation degree (DD), was calculated as represented by Equation 3.3:

$$DD = 100 - \left(\frac{Coll_f}{Coll_0}.100\right)\% \quad (3.3)$$

Where $Coll_0$ (mg) is the collagen content at the beginning of the biogas trials (substrate + sludge) and $Coll_f$ (mg) is the collagen content measured in the final

biomass, both were considered on a dry basis. The collagen content of the substrates and biomass were calculated through determination of the hydroxyproline content (Stegemann, 1958).

Gelatin does not have collagen chains in its structure but it is possible to detect amino acids. For this reason, the hydroxyproline of the substrate and final biomass were analyzed and the degradation of gelatin was measured as degradation of hydroxyproline.

3.3.2.2 Spectroquant cell tests

Spectroquant® Cell Test Kits for quantitative investigation were used to analyse the biomass of the bioreactors photometrically. Ammonium content (Number 1.14739.0001), Chemical Oxygen Demand (Number 1.14541.0001), Iron content (1.14549.0001), Sulfate content (Number 1.14548.0001), and Volatile fatty acids (Number 1.01749.0001) were measured from the biomass samples.

3.3.2.3 High-performance liquid chromatography (HPLC)

Volatile fatty acids (acetic acid, propionic acid, isobutyric acid, butyric acid, and isovaleric acid) were determined by HPLC (Shimadzu prominence Serie 20, equipped with a refractive index detector RID-10A and a photodiode array detector SPD-M20A). Measurements were performed with a mobile phase of 5 mM H_2SO_4, a flow rate of 0.6 mL min^{-1}, a column temperature of 60 °C and a detector temperature of 40 °C. The biomass samples were previously centrifuged at 14,000 rpm for 10 minutes and filtered using a syringe filter holder (filter 0.2 μm pore size) before injection.

3.4 Biogas Production

Biogas production trials were carried out discontinuously and continuously in gastight reactors to assure the absence of oxygen. The substrate, pre-treated or untreated chromium leather waste, and inoculum, sludge with a diversified biocoenosis, were placed inside the reactor. After a sufficient time of contact biogas was produced.

3.4.1 Laboratory scale: batch tests

In this study, laboratory scale batch tests were performed under mesophilic conditions (37 °C ± 2 °C) according to the guideline VDI 4630 (2006) in duplicate or triplicate along with three blanks. The volumes of biogas produced by the blanks (mean of three samples of inoculum without any added substrate) were subtracted from the values obtained for the individual test samples. A t-test was conducted to compare means. As inoculum, mesophilic anaerobic sludge from the local sewage treatment plant was used.

The tests were conducted using glass flasks tightly sealed with a rubber septum (65 mL) and the gas production was measured indirectly on a daily basis using a digital manometer (Leo 3 Keller) (Figure 3.10). The gas volume was calculated from the gas pressure registered and the gas temperature measured. The biogas yield (quantity of generated biogas per quantity of substrate fed) and the biogas formation potential (maximum biogas yield generated from a defined quantity of substrate) are given in norm litres (273 K and 1013 hPa) per kg of organic dry matter of the added substrate (L kg^{-1}). The frequency of measurements was reduced to once every two or three days after a fall in the daily production. Measuring devices for analysing the composition of low biogas amounts (percentage methane and carbon dioxide) were not available.

Fig. 3.10: *Biogas reactors in batch scale and biogas generation measurement.*

The agitation effect was tested in two different systems, with agitation in a shaking water bath (Julabo SW-20C at 150 rpm), and without agitation in a climatic chamber (drying unit Fratelli Carlessi ARMADIO 5B). Volatile fatty acids were injected into the bioreactors in some trials piercing the septum using a syringe. Three different volatile fatty acids were evaluated, acetic acid (Carl Roth, 100% p.a.), propionic acid (SIGMA-ALDRICH, 99.5% ACS), and isobutyric acid (SIGMA-ALDRICH, 99.5% p.a.).

At the end of the process, the resulting biomass was analysed regarding its pH value, water content (DIN EN ISO 4684, 2005), inorganic matter (DIN EN ISO 4047, 1998), chromic oxide content (DIN EN ISO 5398-1, 2007), and collagen content.

3.4.1.1 Two-phase Gompertz model

In many cases, the cumulative biogas production curve can be described as a sigmoidal curve with lag, growth, and asymptotic phases. Therefore, it is possible to use the sigmoidal function of Gompertz, modified as described by Zwietering et al. (1990), to fit the experimental biogas production data. If the cumulative biogas production curve shows a two-phase biogas production, this model needs to be modified to fit the two-phase curve as represented in Figure 3.11 and Equation 3.4.

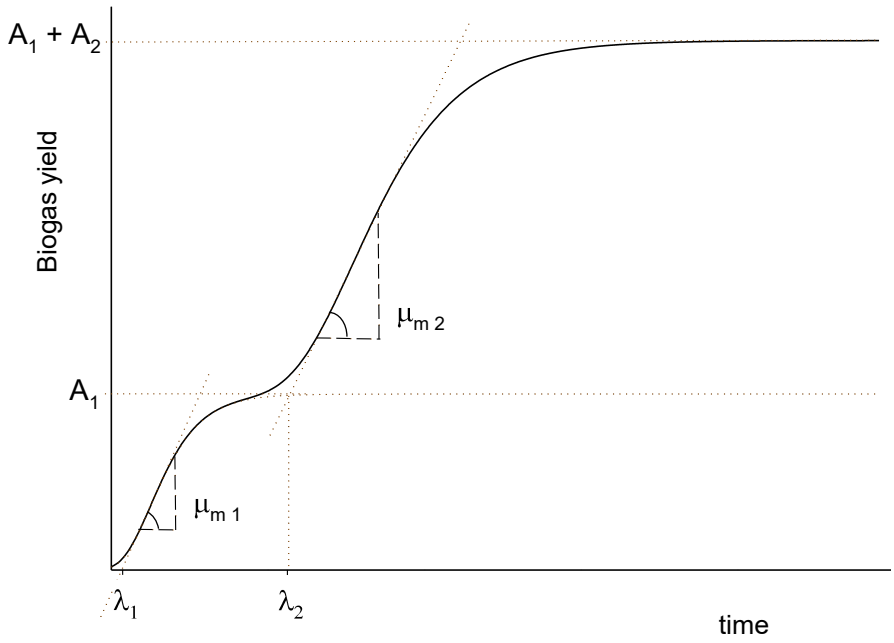

Fig. 3.11: *Two-phase Gompertz model.*

For the two-phase Gompertz model, the duration of the lag-phase (λ_1, d) at the beginning of the anaerobic digestion, the switch to the second phase of the digestion (λ_2, d), the biogas formation of each phase (A_i, L kg^{-1}), and the maximum biogas production rate of each phase of the anaerobic digestion (μ_{mi}, L kg^{-1} d^{-1}) were found with Equation 3.4.

$$y = \sum_{i=1}^{2} \left(A_i \exp\left\{ -\exp\left(\frac{\mu_{mi} e}{A_i} (\lambda_i - t) + 1 \right) \right\} \right) \qquad (3.4)$$

Where y is the cumulative biogas production (L Kg^{-1}), t is the time (days), and e is the Euler's number. The biogas formation potential of the substrate is the sum of the biogas formation of both phases ($A_1 + A_2$). The models were fitted using the software SigmaPlot version 13.0.

3.4.1.2 Substrate to inoculum ratio

According to the guideline VDI 4630 (2006), the substrate should not be overlarge in proportion to the inoculum to prevent inhibition in a batch test. The substrate to inoculum ratio to be respected is represented by Equation 3.5:

$$S/I \leq 0.5 \quad (3.5)$$

Where S is the organic dry matter of the substrate (g) and I is the organic dry matter of the inoculum in the reactor (g). In order to investigate the possibility of different loads, batches were tested using a substrate to inoculum ratio higher, lower, and approximately equal to 0.5.

3.4.2 Pilot scale: continuous tests

Continuous anaerobic digestion tests were performed according to the guideline VDI 4630 (2006). The apparatus consisted of a 20 L gastight stirred tank with infeed and outlet, and a gas offtake connection to collect the formed biogas (Figure 3.12). The temperature was kept under mesophilic conditions (37 °C ± 2 °C) using a circulation thermostat (Huber CC-202C) and the biomass was constantly mixed at 50 rpm using a paddle stirrer (Heidolph RZR 2041). The biogas formation in norm litres per kg of sludge (L kg^{-1} d^{-1}) was measured daily using a drum-type gas meter (Ritter TG05/5), and the quality of the gas as well as the hydrogen sulfide concentration were measured using an electronic analyser (OPTIMA 7 BIOGAS) once every two days. The hydrogen sulfide concentration was controlled by adding iron (III) chloride hexahydrate (Merck) which works as an H_2S scavenger, if necessary. Mesophilic anaerobic sludge from the SÜDLEDER tannery (Rehau, Germany) was used as inoculum. Because of its source the sludge was also analysed regarding its chromium and collagen content.

At the beginning of the test the reactor was filled with approximately 20 kg of inoculum (wet basis). Substrate was added once every two days excluding weekends via a dip tube located at the head end of the reactor, starting at a loading rate of substrate per kg of sludge of 0.5 g kg^{-1} d^{-1} (substrate mass in organic dry matter). After the daily methane production was constant, the loading rate was raised by 0.5 units and the process was repeated until the biogas production stopped to increase.

Fig. 3.12: *Continuous anaerobic digestion test apparatus (source: MyFerm I manual – Landgraf Laborsysteme HLL GmbH).*

Once a week a sample of biomass was taken for characterization regarding its pH value, water content (DIN EN ISO 4684, 2005), inorganic matter (DIN EN ISO 4047, 1998), chromic oxide content (DIN EN ISO 5398-1, 2007), and collagen content through the determination of the hydroxyproline content. The biomass was also analysed photometrically using Spectroquant® Cell Test Kits and chromatographically to determine its volatile fatty acids content.

3.4.2.1 Chemical oxygen demand (COD) balance

The biogas formation measurements and the methane quantity were used to calculate the substrate degradation through a COD balance with Equation 3.6 (VDI 4630, 2006). Using this method it is possible to determine the degradation level of the substrates through biogas production (yield and gas composition) and the substrate quantity fed data.

$$\text{COD degree of degradation} = \frac{V_{Gas} \cdot x_{CH4}}{320.m_{substrate} \cdot COD_{substrate}} \% \quad (3.6)$$

Where V_{Gas} is the volume of biogas in mL d^{-1} (norm), x_{CH4} is the amount of methane, $m_{substrate}$ is the substrate addition in g d^{-1} (organic dry matter), and $COD_{substrate}$ is the substrate's chemical oxygen demand in g_{O2} g^{-1} (organic dry matter).

As explained in the guideline VDI 4630 (2006), this method is based on the oxygen needed to oxidize the produced methane. From stoichiometry it is known that 2 moles of O_2 (64 g) are necessary to oxidize 1 mole of methane, meaning that 1 mole of CH_4 corresponds to a COD of 64 g. As 1 mole of gas methane corresponds to 22.4 L (ideal gas law) the value of 350 mL_{CH4} g_{O2}^{-1} was reached but, as about 10% of the converted COD is for the reformation of biogas, this means about 320 mL_{CH4} g_{O2}^{-1}. It is possible to have the COD of the produced methane using this value, the quantity of methane, and the added substrate. The portion of CO_2 can be ignored because this carbon is already oxidized.

The COD of the substrate was calculated for bovine hide collagen with the stoichiometry of Equation 3.7 (VDI 4630, 2006) and data from the UniProt database (The UniProt Consortium). This value represents the chemically oxidisable material, the maximum of energy that could be recovered by biogas (Drosg et al., 2013).

$$C_aH_bO_cN_d + \left(2a + \frac{b}{2} - c - \frac{3d}{2}\right)O \rightarrow aCO_2 + \frac{b-3d}{2}H_2O + dNH_3 \quad (3.7)$$

3.4.3 Possible Sources of Errors

There are many different sources of error in an experiment, regardless of the level of precision in which the experiments were carried out. Methods and equipment used in this study present errors and have influence in the final results. The used techniques comprise physical, chemical, biochemical and biological test.

The equipment used to measure the production of biogas present errors in their measurements as specified by the manufacturer. In batch trials, the digital manometer Leo 3 has an error lower than 0.1% of full scale with a measuring range of pressure of 0 to 4 bar. For the continuous trials, the electronic gas analyser OPTIMA 7 BIOGAS has an absolute error of 0.5% or 5% of the measured value of CH_4 and CO_2, whichever is larger.

Chemical analyses have errors even when carefully performed. For example, each Spectroquant® Cell Test Kit has a different error. The Ammonium content and Iron content kits have an absolute error of 0.05 mg L^{-1}. The Chemical Oxygen Demand kit has an absolute error of 29 mg L^{-1}. The Sulfate content kit has an absolute error of 8 mg L^{-1}. Finally, the Volatile fatty acids kit has an absolute error of 69 mg L^{-1}.

The error of the determination of the hydroxyproline content is 1.3% of rate. However, when the determination of the collagen content is necessary the value has to be multiplied by the factor 7.46 and the error of the determination of the collagen content is 9.7% of rate. Chemical analyses were also performed in duplicate or triplicate, with the exception of the Spectroquant® Cell Test Kit, which were performed only once because of the limited amount of biomass available for the analyse.

To ensure the quality of the biogas results, the batch trials were performed in duplicate or triplicate. Continuous biogas trials could not be repeated because trials were very time-consuming however the courses of the measured values were carefully checked regarding plausibility. Nevertheless biological tests show the highest variability because of a limited definition and characterization of the sludge as a living system.

4 Results and Discussion

The present section illustrates chemical analyses and biogas production results for the extruded shavings and offcuts, shavings treated hydrothermally, and autoclaved shavings. Results for untreated chromium shavings and offcuts, and for gelatin are shown for comparison purposes.

4.1 Chemical analyses of the pre-treated samples

Untreated chromium shavings and offcuts, and pre-treated shavings and offcuts were evaluated using different chemical analyses to determine their susceptibility to anaerobic degradation and get a hint on the effectiveness of the pre-treatments.

4.1.1 Characterization of the pre-treated samples

The characterization of the pre-treated samples gives important information for the biogas trials. Only organics are capable of producing biogas and, therefore, it is important to quantify the organic and inorganic content of the studied substrates. The characterization of the pre-treated shavings and offcuts, untreated chromium shavings, untreated leather offcuts, and gelatin is shown in Table 4.1.

With the exception of hydrothermal treatment, the water content was reduced by pre-treatments. Part of the non-bonded water of the chromium shavings and leather offcuts is evaporated due to the high pre-treatment temperatures part resulting in a drop of the pre-treated sample's water content.

Tab. 4.1: *Characterization of the pre-treated and untreated samples.*

	Water content (%)	Organic matter (%)*	Hydroxyproline content (%)*	Chromium content (%)*,**
Gelatin	90.5 ± 0.0	99.0 ± 0.0	13.1 ± 0.2	-
	Water content (%)	**Organic matter (%)***	**Collagen content (%)***	**Chromium content (%)*,****
CS	19.7 ± 0.1	88.8 ± 0.1	77.0 ± 0.6	4.6 ± 0.0
Offcuts	11.4 ± 0.0	93.6 ± 0.0	n.d.	4.1 ± 0.1
E100W	45.2 ± 0.7	88.9 ± 0.1	72.6 ± 2.4	n.d.
E130W	25.8 ± 0.2	89.0 ± 0.1	77.8 ± 1.1	n.d.
E150W	10.6 ± 0.2	89.1 ± 0.0	79.1 ± 0.3	n.d.
E170W	6.3 ± 0.1	88.9 ± 0.1	76.8 ± 0.7	4.4 ± 0.0
E100D	15.1 ± 0.1	88.9 ± 0.0	74.2 ± 0.9	4.6 ± 0.0
E130D	4.4 ± 0.0	88.7 ± 0.1	75.4 ± 0.7	n.d.
E150D	3.7 ± 0.1	88.8 ± 0.1	74.5 ± 1.0	n.d.
E170D	2.5 ± 0.1	88.9 ± 0.1	73.0 ± 1.7	n.d.
EO	3.9 ± 0.1	93.0 ± 0.1	50.0 ± 0.5	3.9 ± 0.0
H140	83.1 ± 0.1	88.7 ± 0.1	73.7 ± 1.3	4.4 ± 0.0
H150	83.6 ± 0.1	88.9 ± 0.2	74.7 ± 0.3	4.4 ± 0.0
H170	85.3 ± 0.3	89.0 ± 0.0	76.6 ± 1.2	4.3 ± 0.0
A3	11.8 ± 0.9	n.d.	n.d.	n.d.
A6	12.2 ± 0.2	n.d.	n.d.	n.d.
A12	11.3 ± 0.1	n.d.	n.d.	n.d.
A24	11.0 ± 0.3	n.d.	n.d.	n.d.
A48	10.6 ± 0.0	n.d.	n.d.	n.d.
A96	10.3 ± 0.3	n.d.	n.d.	n.d.
A192	9.4 ± 0.5	n.d.	n.d.	n.d.
A384	10.9 ± 0.2	n.d.	n.d.	n.d.

*Dry basis; mean ± standard deviation, n = 3
**Measured as chromium oxide
n.d. – not determined

The organic matter in the samples remains the same after pre-treatments, about 93% for leather offcuts and 89% for chromium shavings. This is important because the organics must be preserved for producing biogas in the anaerobic digestion. The extruded offcuts and untreated offcuts have a higher content of

organics because of all the chemicals added during the wet end and finishing process, such as dyes, retanning agents, fatliquors, and pigments (Covington, 2009).

The collagen content is also barely unchanged after pre-treatment. This protein is the main component of the chromium leather waste. Only about 12% of the chromium shavings are a different type of organics, for instance fats. Therefore, collagen is the most important parameter to calculate the substrate degradation after anaerobic digestion. The collagen content of the extruded offcuts is 50% only. A large part of the organics is from other sources and could have a very low degradability.

Almost half of the inorganic part of the samples is chromium oxide. Chromium also remains the same after pre-treatment. Other inorganics in the samples result from the chemicals used in tanneries.

The autoclaved shavings were produced on a very small scale and, therefore, most of the characterization parameters were not measured. The pre-treated sample available was not sufficient for the experiments.

4.1.2 Scanning electron microscopy

Chromium shavings and leather offcuts were compared with the different pre-treated samples using SEM images. Figure 4.1a shows the untreated chromium shavings. A fibrous structure can be seen, the fibrils closely aligned together forming the fibres. Similarly, the leather offcuts show an intact fibrous structure (Figure 4.1b).

Not all SEM images were shown for length and clarity. However, the SEM images for the pre-treated chromium leather waste not illustrated in this section can be seen in the Appendix (Figure 5.1).

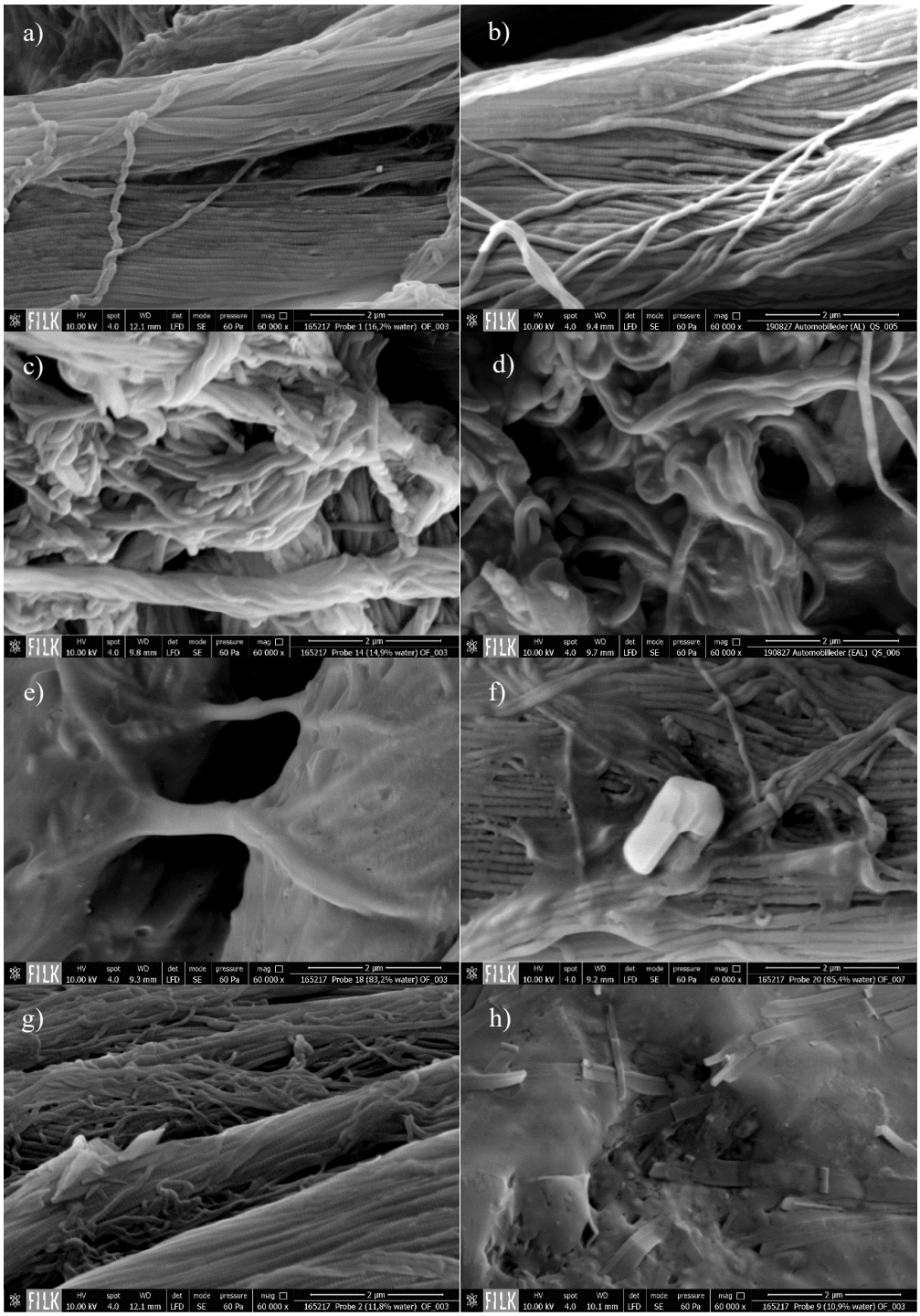

Fig. 4.1: *SEM images of chromium shavings (a), leather offcuts (b), shavings extruded dry at 100 °C (c), extruded leather offcuts (d), shavings treated hydrothermally at 140 °C (e), shavings treated hydrothermally at 170 °C (f), autoclaved over a period of 3 minutes (g), and autoclaved over a period of 384 minutes (h) at 60,000 × magnification.*

Figure 4.1c shows the shavings extruded dry at 100 °C and Figure 4.1d the leather offcuts extruded at 170 °C. Severe damage to the fibrous structure can be seen for both samples, fibrils are randomly positioned and the structure appears to be shrunken. Since the collagen molecules were organized as tissue, the fibres shrink when exposed to temperatures higher than the denaturation temperature (Meyer, 2019). An organized fibrous structure, as identified for the untreated chromium shavings and leather offcuts, cannot be seen anymore. Similar behaviour was verified before by Meyer et al. (2005) using SEM images. The authors observed a fibrillar structure in the initial collagenous material, a parchment-like raw material from limed cattle hides, but after thermomechanical treatment in an internal mixer (thermo-mechanical treatment) this structure disappeared.

The shavings treated hydrothermally at 140 °C and 170 °C (Figures 4.1e and 4.1f) show a rather deteriorated structure. The high temperatures, which the chromium shavings had been exposed to, started a melting process of the fibres. However, the melting appears to be superficial and the randomly positioned fibres are still present. In Figure 4.1e, the captured surface appears to be molten but the pattern of the fibres can still be seen, indicating superficial melting. In Figure 4.1f, molten areas can be seen in the lower part of the capture but fibres are also clearly visible. The presence of molten areas is a strong indicator for the fact that the samples are denatured.

The shavings autoclaved over a period of three minutes (Figure 4.1g) are very similar to the untreated chromium shavings (Figure 4.1a), however fibrils on the surface are detached from the main structure, probably due to initial shrinking. The shavings autoclaved over a period of 384 minutes (Figure 4.1h) were pre-treated for a sufficient time to denature the chromium shavings and the surface of the material appears to be molten.

Additionally, for the shavings treated hydrothermally at 170 °C (Figure 4.1f), formation of crystals was identified after the hot liquid sample generated by hydrothermal treatment had been solidified. Crystals were also found for other extruded shavings (Figure A.1). In order to identify the observed crystals, chromium shavings, shavings extruded dry at 100 °C, shavings extruded wet at

170 °C, shavings treated hydrothermally at 150 °C, and shavings treated hydrothermally at 170 °C were analysed using energy-dispersive X-ray spectroscopy (EDX). Results are shown in Figure 4.2.

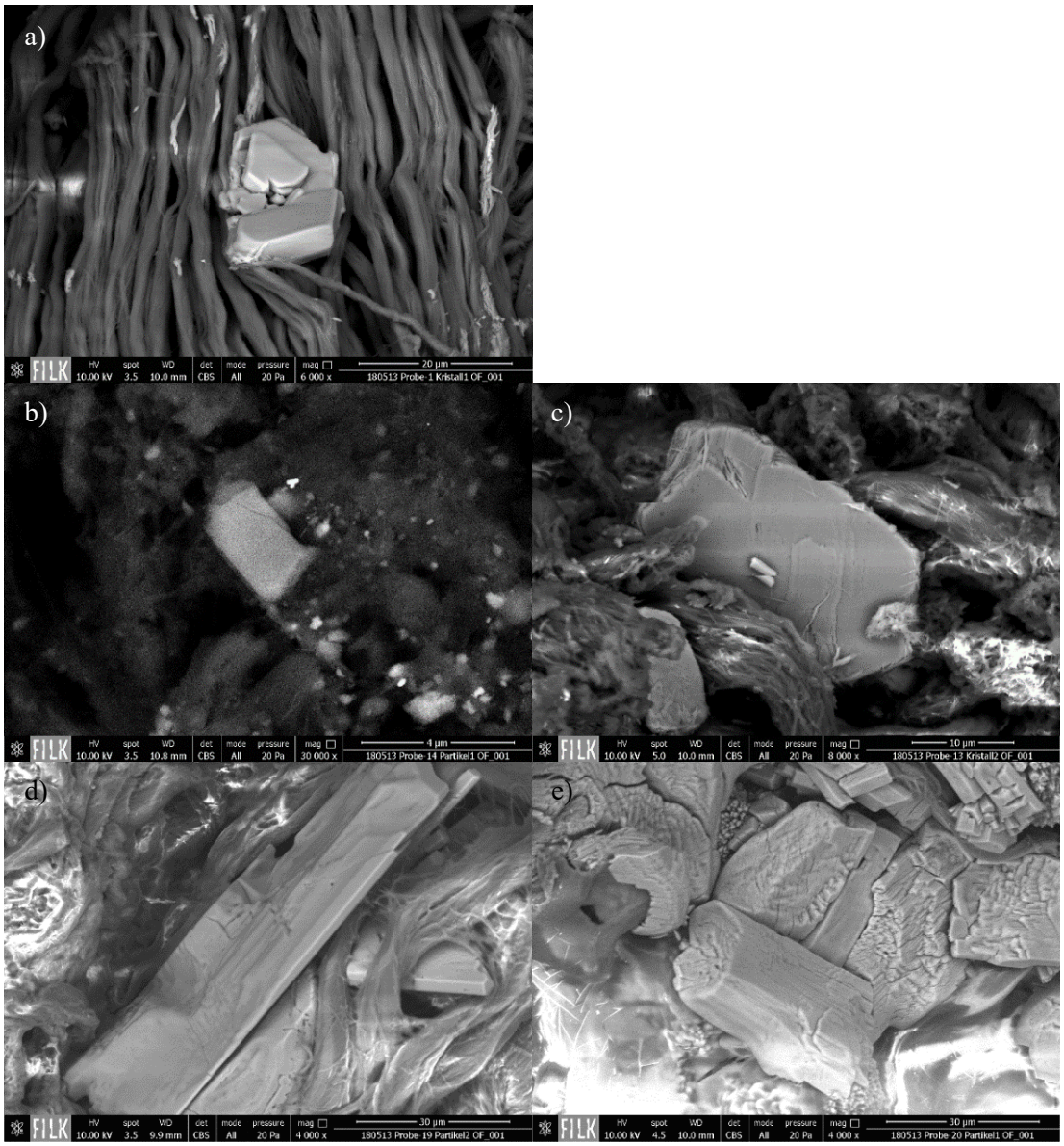

Fig. 4.2: *SEM images for EDX of chromium shavings (a), shavings extruded dry at 100 °C (b), shavings extruded wet at 170 °C (c), shavings treated hydrothermally at 150 °C (d), shavings treated hydrothermally at 170 °C (e) at different magnifications.*

Formation of crystals was observed for all analysed samples and it is presumed that these crystals can be found on the surface of all samples. The elements present in the crystals were analysed using EDX. Results revealed that these crystals are mainly composed of calcium (about 20%), sulfur (about 15%) and oxygen (about 65%). Consequently, these crystals could be $CaSO_4$ crystals, which possibly originated from the basic chromium sulfate used in the tanning process to produce leather. The shape of the crystal observed in the SEM images is also compatible with the $CaSO_4$ observed by Sirota et al. (1992).

4.1.3 Differential scanning calorimetry and enzyme assays

Specific enthalpy found in DSC analyses and degradation by trypsin results for the extruded shavings and offcuts (Figure 4.3a), the shavings treated hydrothermally (Figure 4.3b), and autoclaved shavings (Figure 4.3c) are compared to results for the untreated chromium shavings. The error bars represent the standard deviation for the experimental data. Analysis of the results led to the conclusion that the triple helical structure in almost all samples could be denatured due to the pre-treatments. DSC results show that, with exception of the shavings autoclaved over a period of three minutes, there is no enthalpy or hardly any enthalpy for the pre-treated samples. Therefore, no hydrogen bonds stabilizing the triple helix remain.

Even though collagen in the samples was denatured due to the pre-treatments, the hydroxyproline present in the triple helix chains appears to be intact. This means that the pre-treatment opens up the triple helix structure without destroying the chains. This can be seen in the collagen content of the samples (Table 4.1), which is very similar for untreated and pre-treated samples. The method used to measure the collagen content is also used to determine the content of the amino acid hydroxyproline (Stegemann, 1958), which is almost the same before and after pre-treatment.

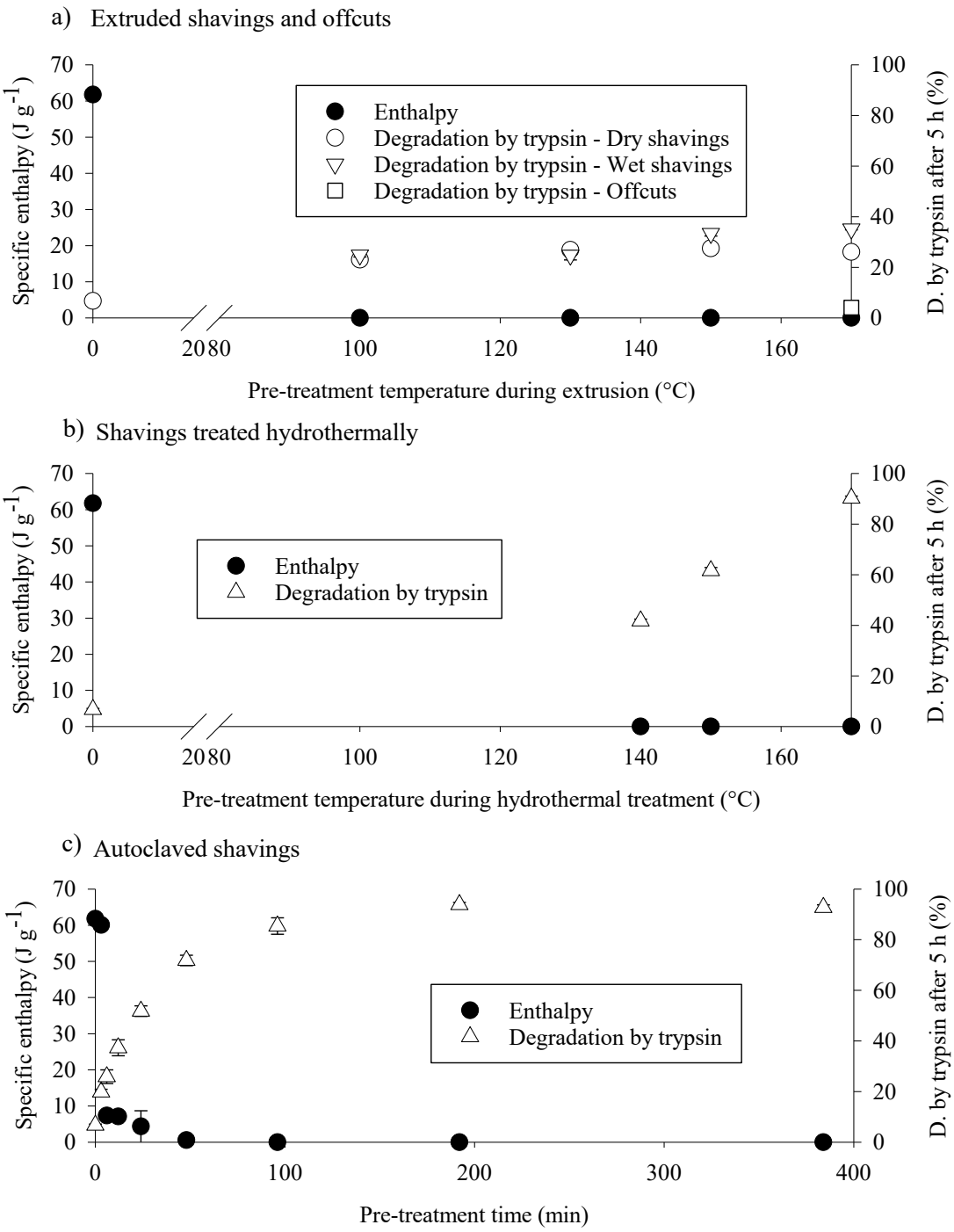

Fig. 4.3: *Enthalpy of the denaturation process and degree of degradation by trypsin of extruded shavings and offcuts (a), shavings treated hydrothermally as a function of the pre-treatment temperature (b), and autoclaved shavings as a function of the pre-treatment time (c) and chromium shavings.*

Trypsin assays show that trypsin can degrade the chromium shavings more easily if they were pre-treated. Trypsin was able to degrade the chromium shavings and pre-treated samples, which contain more than 4% chromium oxide (Table 4.1). Consequently, this quantity of chromium is not toxic for trypsin. It can be assumed that the levels of chromium content in the studied chromium leather waste are lower than what would be toxic for other enzymes in the anaerobic digestion.

Figure 4.3a reveals that using extrusion as a pre-treatment it is possible to increase the susceptibility of the chromium shavings to be degraded by trypsin. The degradation increases from 6.7 ± 0.4% up to 35.2 ± 0.4% when using shavings extruded wet at 170 °C instead of untreated chromium shavings. Results showed an increasing tendency of degradation with increasing extrusion temperature. Degradation levels for the previously moistened extruded samples for the temperatures 150 °C and 170 °C were slightly higher than for the shavings extruded dry at the same temperatures. The means of digestibility were compared by using the t-test. There was no statistically significant difference between samples extruded dry and wet at 100 °C ($p = 0.057$), and extruded dry and wet at 130 °C ($p = 0.257$), but there was a difference between samples extruded dry and wet at 150 °C ($p = 0.010$), and dry and wet at 170 °C ($p = 0.004$). The comparison between samples extruded dry at 100 °C and wet at 170 °C showed a statistically significant difference ($p = 5.10^{-6}$).

With regard to the extruded offcuts, the sample had a very low degradation by trypsin value (4.1 ± 0.3%), even lower than the degradation value obtained for untreated chromium shavings (6.7 ± 0.4%). Leather offcuts were treated in numerous steps after tanning unlike chromium shavings. The wet end and finishing process in the tannery industry made this chromium leather waste even more inaccessible for anaerobic degradation and insoluble with the addition of a variety of polymers during the process.

The results of degradation by trypsin for the shavings treated hydrothermally showed a clear increase when using higher pre-treatment temperatures. The shavings pre-treated at 170 °C were highly accessible for trypsin reaching the highest degradation level among the studied samples, 90.3 ± 0.8% (Figure 4.3b).

The autoclaved shavings (Figure 4.3c) showed high degradation even with short pre-treatment time. After three minutes of thermal pre-treatment only, the degradation of the waste went from 6.7 ± 0.4% (untreated chromium shavings) to 19.8 ± 1.0% and within only 24 minutes it was possible to have 51.8 ± 2.1% of degradation reaching more than 90% after 192 minutes. After 192 minutes of heat treatment, degradation reached a plateau and any longer autoclaving process would not be reasonable.

Additionally, the untreated chromium shavings and the shavings extruded dry at 100 °C were tested for degradation by collagenase to evaluate if this enzyme, capable of destabilizing collagen (Meyer, 2019), would be more effective to degrade untreated and pre-treated chromium shavings. The *Clostridium histolyticum* has the ability to produce collagenase and belongs to the bacteria clostridia, the main bacteria found in sludge (McInerney, 1988). *Clostridium histolyticum* is probably present in the medium producing collagenase. Therefore, this enzyme could play an important role in the degradation of the studied substrates. Results of degradation by collagenase of the untreated chromium shavings, shavings extruded dry at 100 °C, and white hide powder, untanned hide used as reference, are shown in Figure 4.4. The error bars represent the standard deviation for the experimental data.

Even with longer times of contact between substrate and enzyme, collagenase did not digest the untreated chromium shavings effectively. Figure 4.4 shows that degradation of the untreated chromium shavings reached a value of about 12% after one day and it remains the same after seven days. The white hide powder was quickly hydrolyzed by the collagenase. This was to be expected because untanned hides are prone to degradation by proteases. Degradation of the shavings extruded dry at 100 °C increased linearly with the time of contact, reaching up to 86% of degradation after seven days which is almost the degradation degree of the white hide powder. Pre-treatment of the chromium shavings was necessary to enable their degradation by collagenase, which runs slower than for the white hide powder.

Results published by Covington (2009) also suggest that the untreated chromium shavings cannot be easily degraded by proteases. Protease assays

showed that the enzymes had no visible effect on chromium leather samples unless the concentration of enzyme is abnormally high, the pH value is close to 9, and the temperature of the reaction is close to 50 °C. Under these conditions no effects are observed up to the point the chromium-tanned leather is suddenly and completely degraded.

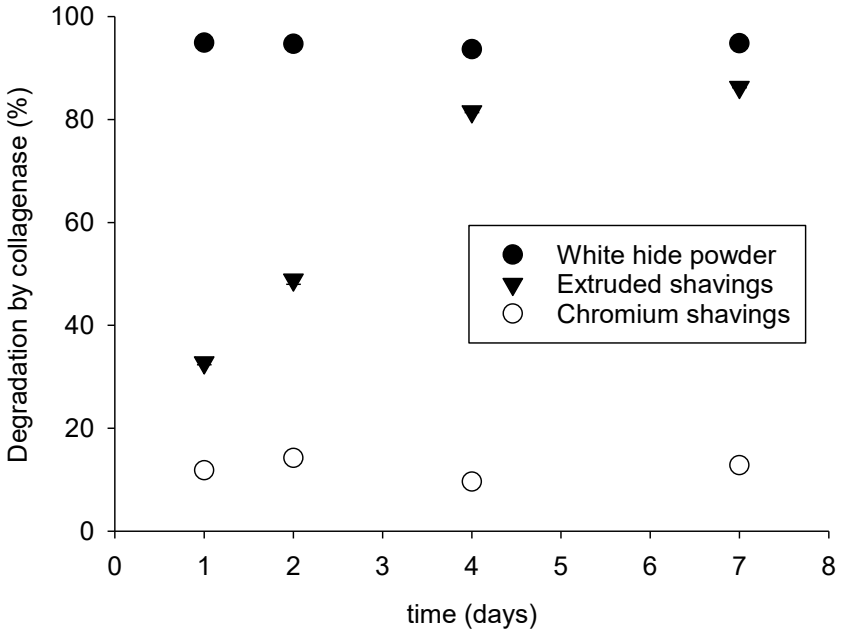

Fig. 4.4: *Degree of degradation by collagenase of white hide powder, untreated chromium shavings, and shavings extruded dry at 100 °C as a function of time.*

Additionally, Meyer (2019) agrees that a pre-treatment can ease enzymatic degradation of tanned hides. Under different conditions, degradation of collagen was tested with different enzymes. Porcine hides with and without synthetic cross-links (tanning effect achieved by hexamethylendiisocyanate) were degraded by enzymes, such as trypsin and collagenase. Synthetic cross-links suppressed the enzymatic action of all enzymes tested. Enzymatic degradation of the tanned structure was only possible when collagen was denatured (pre-treated by heat). However, collagenase is much more effective for degradation of the pre-treated collagen than trypsin.

Results demonstrated that almost all samples were completely denatured by the pre-treatments tested. Moreover, chromium shavings are more easily accessible to degradation by proteases after pre-treatment than without pre-treatment. Neither trypsin nor collagenase were able to effectively degrade untreated chromium shavings. The higher the pre-treatment temperature the easier the sample degradable by trypsin. However, the wet end and finishing process prevents accessibility to trypsin even after the leather offcuts were pre-treated by extrusion. The quantity of chromium present in the chromium leather waste was not toxic to trypsin and collagenase.

4.1.4 Solubility in water

Figure 4.5 shows the results of solubility in water at a temperature of 37 °C for the pre-treated samples and the untreated chromium shavings. The error bars represent the standard deviation for the experimental data.

Figure 4.5a shows that the extruded shavings and the untreated chromium shavings had very similar values of solubility in water at a temperature of 37 °C. Despite the visual similarities, the solubility results for most of the extruded shavings showed statistically significant differences when compared to untreated chromium shavings using the t-test. An exception were the shavings extruded dry at 130 °C ($p = 0.529$). In general, solubility was slightly reduced by pre-treatment, probably due to some additional cross-links generated by extrusion (Klüver and Meyer, 2013).

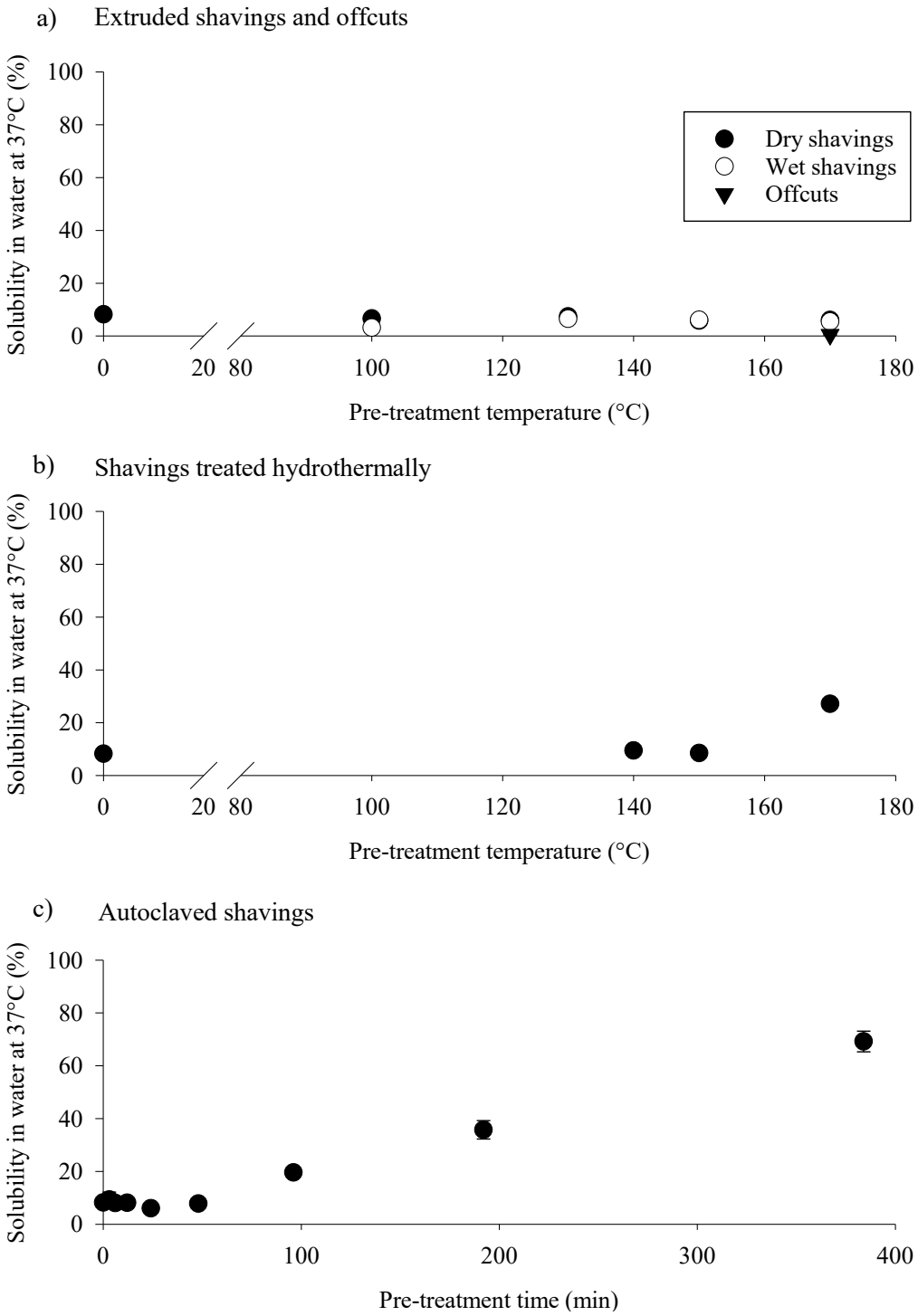

Fig. 4.5: *Solubility in water at a temperature of 37 °C of extruded shavings and offcuts (a), shavings treated hydrothermally as a function of the pre-treatment temperature (b), and autoclaved samples as a function of the pre-treatment time (c) and chromium shavings.*

Gorham et al. (1992) studied the formation of cross-links on dry collagen treated thermally (heat cured), and concluded that intermolecular amide bonds were formed. The cross-linking mechanism involves the reaction of aspartate and glutamate residues with the amino groups of lysine and hydroxylysine. They also found a reduction in solubility and an increase in degradation by trypsin but in their case the solubility reduction was more significant because the samples were dried and the presence of moisture in the samples accelerated the rate of denaturation with heat. However, the reduction in solubility verified for the extruded shavings was very low. The values obtained were also very similar for chromium shavings treated at the same temperature with or without humidification process. The only extruded shavings that showed significant differences were the shavings extruded dry and wet at 100 °C ($p = 0.019$).

The hydrogen bonds that stabilize the triple helices could be broken down by extrusion pre-treatment which also enabled degradation of the chromium shavings as can be seen from the DSC results. However, no chromium shavings could be hydrolyzed and no gelatin generated by extrusion. Consequently, solubility in water could also not be increased by extrusion pre-treatment. The transformation of collagen-based materials into gelatin depends on exposing the material to a temperature higher than the denaturation temperature and breaking down several bonds to tear apart the triple helix structure. To degrade the samples, high temperatures and mechanical forces are used for extrusion pre-treatment. However, it was not possible to generate gelatin. This is probably due to the fact that the time of contact of the samples with the heated extruder, approximately three minutes, is not sufficiently long to transfer the collagen molecules into gelatin chains, but it was possible of shrink them causing the small reduction in solubility.

Extruded offcuts (Figure 4.5b) showed the lowest solubility among the tested samples, 0.5 ± 0.2%, even lower than that of untreated chromium shavings. This low value is a consequence of the wet end and finishing process in tanneries, during which chromium-tanned leather is processed to produce finished leather. It is not possible to revert this effect by extrusion pre-treatment, and the material remains almost insoluble.

The solubility of the shavings treated hydrothermally (Figure 4.5b) can be related to the fibrous structure of the samples and the reduction of protein chain lengths. The solubility of the shavings pre-treated hydrothermally at 170 °C, which had liquid consistency immediately after the pre-treatment, increased from 8.2 ± 0.3% (untreated chromium shavings) to 27.1 ± 0.9%. However, compared to soluble gelatin this value is still low. Therefore, the collagen was not completely transformed into gelatin. The solubility of the shavings treated hydrothermally at 140 °C and 150 °C, 9.4 ± 0.9% and 8.4 ± 0.6% respectively, showed no statistically significant difference when compared to the untreated chromium shavings for which the t-test was applied ($p = 0.197$ and $p = 0.640$, respectively). As observed in SEM, melting of the structure caused by hydrothermal treatment was superficial and the samples still show a partially fibrous structure (Figure 4.1e and 4.1f).

The solubility of the autoclaved shavings (Figure 4.5c) increases with longer pre-treatment times. The shavings treated for more than 96 minutes, that is those which showed melting of the fibrous structure in some areas, had a linear growth trend of solubility with regard to the pre-treatment time. With three minutes of autoclaving pre-treatment the results of solubility in water are very similar to the result of untreated chromium shavings because the pre-treatment time was insufficient to degrade the chromium shavings. In other words, the longer the pre-treatment time the higher the solubility in water, and it can also be concluded that the degree of degradation of the shavings is higher.

In general, solubility in water of the chromium leather waste was not affected by the pre-treatments. Chromium shavings treated hydrothermally at 170 °C and shavings autoclaved for more than 96 minutes were the exception. However, even in these cases the solubility is low compared to that of gelatin. The extruded offcuts showed the lowest solubility due to the wet end and finishing process.

The chemical analyses used to evaluate the pre-treated chromium leather waste show that all extruded shavings and shavings treated hydrothermally, and most autoclaved shavings are denatured. The chromium shavings are more easily accessible to degradation by trypsin and collagenase after pre-treatment.

However, for most of the pre-treated shavings solubility does not change. Extruded leather offcuts show a different behaviour with lower values of degradation by trypsin and lower solubility than untreated chromium shavings.

4.2 Biogas Production

The biogas and methane production of the pre-treated chromium leather waste was investigated through batch and continuous biogas production trials to prove feasibility of using pre-treated samples to produce biogas. Autoclaved shavings were produced on a small scale. Screw cap micro tubes (2 mL) were used as autoclave apparatus because the use of a laboratory autoclave does not allow for precise control of the pre-treatment time. Therefore, testing the autoclaved shavings in biogas trials was unviable.

4.2.1 Laboratory scale: batch tests

The biogas formation potential was measured in batch tests for untreated chromium shavings, pre-treated samples, and gelatin.

4.2.1.1 Comparison of different agitation methods

Initially, two samples pre-treated with extrusion and the untreated chromium shavings were tested for biogas production. Batches were tested with agitation in a shaker water bath and without agitation in a climatic chamber. The shavings extruded dry at 100 °C and extruded wet at 170 °C were selected as they represent the extremes of the extrusion pre-treatment, the former being the sample with the lowest degree of treatment, and the latter the highest degree of treatment. The untreated chromium shavings were tested for comparison purposes. Trials with the same substrate had the same substrate to inoculum (S/I) ratio in the batch with and without agitation. The two-phase Gompertz model was used to fit the experimental data for a better representation of the hydrolysis and lag-phase of the anaerobic digestion of the tested substrates. Results are

shown in Figure 4.6 and Table 4.2. The error bars in Figure 4.6 represent the standard deviation for the experimental data.

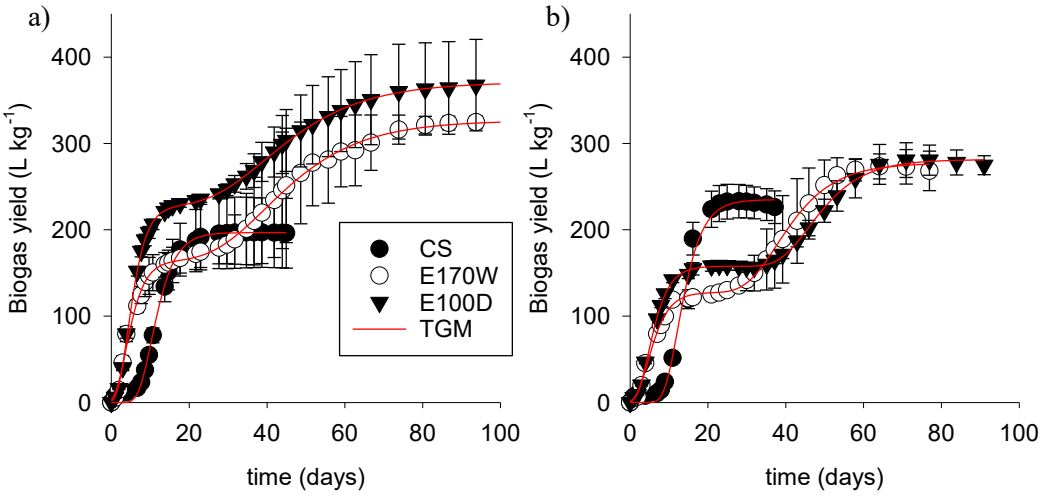

Fig. 4.6: *Cumulative biogas production and two-phase Gompertz simulation (red line) for chromium shavings (S/I = 2.6), shavings extruded wet at 170 °C (S/I = 3.2), and dry at 100 °C (S/I = 2.9) in agitated bioreactors (a) and in non-agitated bioreactors (b).*

Tab. 4.2: *Parameters of biogas production from the two-phase Gompertz equation.*

	λ_1 (d)	μ_{m1} (L kg^{-1} d^{-1})	A_1 (L kg^{-1})	λ_2 (d)	μ_{m2} (L kg^{-1} d^{-1})	A_1+A_2 (L kg^{-1})	R^2
Non-agitated							
CS	8.8	24.5	235.0	∞	-	-	0.9907
E100D	1.8	20.0	157.8	38.3	5.9	281.4	0.9989
E170W	1.5	15.6	127.6	29.7	6.8	277.2	0.9980
Agitated							
CS	6.9	21.3	196.7	∞	-	-	0.9969
E100D	1.6	31.7	228.2	25.2	3.7	370.9	0.9996
E170W	1.0	22.1	164.4	25.9	4.4	326.6	0.9979

The modified Gompertz model has been widely used in the simulation of biogas and methane production results (Li et al., 2011; Kafle and Kim, 2013b; Chanakya et al., 2015; Kim and Kim, 2017). This model can predict the lag-phase of the process, a period associated with the hydrolysis of the substrate in the digester and presents a good fit to experimental results (Kafle and Kim, 2012;

Kafle and Kim, 2013a; Li et al., 2016; Rajput et al., 2018). However, it was necessary to adapt the equation to fit biogas production curves with two phases creating a two-phase Grompertz model (Equation 3.4).

The adapted equation was able to fit biogas production curves with one or two phases. For all tested conditions the coefficient of determination (R^2) is higher than 0.99 indicating that the model fitted well the experimental data. Untreated chromium shavings had a one-phase biogas production curve. This is indicated in the two-phase Grompertz model with high values of λ_2 or an A_2 of almost 0. A comparison between results of batches with and without agitation indicated that agitation reduces the lag-phase of the anaerobic digestion for untreated and pre-treated shavings (Table 4.2) and consequently the hydrolysis phase. Regarding the maximum biogas production rate of the first phase, values increased for the extruded shavings but not for the untreated chromium shavings. The agitation of the reactors eases the mass transfer in the bioreactors and prompted contact between substrate and bacteria increasing the production rate. The agitation of the reactors favours the biogas production for the extruded shavings and it is expected that the same would occur for all pre-treated samples.

The degradation degree of collagen of each trial was estimated after the characterization of the final biomass in the reactor. Considering the content of collagen of each substrate (Table 4.1) and using a mass balance, the collagen content at the beginning of the trials (combination of inoculum and substrate) can be calculated. Using this value and the collagen content of the final biomass in the reactor with Equation 3.3 it was possible to calculate the degradation degree of collagen achieved by the process. Table 4.3 shows the characterization of the final biomass and the degradation degree of collagen in the process.

The characterization of the final sludge enabled the estimation of the degradation degree of collagen (Table 4.3). Results with and without agitation were very similar. The extruded samples showed a degradation degree of collagen above 99%, a very high destruction of the collagen of the original substrate and much

higher than the degree reached by the untreated chromium shavings. This indicates that chromium shavings are a complex substrate and the pre-treatments are important to assure the reduction of the final waste.

Tab. 4.3: *Biomass characterization after digestion and degradation degree of collagen for agitated and non-agitated bioreactors.*

	Collagen (%)*	Degradation degree (%)	Chromium (%)**
Non-agitated			
CS	29.9 ± 3.4	51.4	2.1 ± 0.1
E100D	0.5 ± 0.1	99.5	2.1 ± 0.0
E170W	0.4 ± 0.1	99.6	3.1 ± 0.3
Agitated			
CS	32.1 ± 1.3	44.7	2.2 ± 0.0
E100D	0.4 ± 0.0	99.5	2.2 ± 0.1
E170W	0.2 ± 0.0	99.8	3.9 ± 0.5

*Dry basis; mean ± standard deviation, n = 3

**Dry basis; mean ± standard deviation, n = 2, measured as chromium oxide

Comparison of the biogas formation potential results of batches with and without agitation indicated that agitation favours the biogas production for the pre-treated samples. The shavings extruded wet at 170 °C had a biogas formation potential of about 327 and 277 L kg^{-1} in the trials with and without agitation, respectively. The shavings extruded dry at 100 °C had a biogas formation potential of about 371 and 281 L kg^{-1} (Table 4.2). This means an increase of biogas formation potential of almost 20% for the first substrate when comparing the agitated with the non-agitated batches. The biogas formation potential of the untreated chromium shavings in both batches were very similar, about 235 L kg^{-1} for the non-agitated and 197 L kg^{-1} for the agitated batch.

The use of agitation could not only reduce the lag-phase for the chromium shavings but also improve every aspect of the biogas production using extruded chromium shavings. The agitation of the system facilitates the mass transfer in the bioreactors and the contact between substrate and bacteria. Therefore, all the following biogas production trials were carried out using agitation in a shaker water bath.

4.2.1.2 Comparison of different pre-treatments

The biogas yield was measured in batch tests with untreated chromium shavings, gelatin, shavings extruded at different temperatures and humidity conditions and shavings treated hydrothermally at different temperatures. The different substrates were exposed to anaerobic digestion with a substrate to inoculum ratio of approximately 0.5 to investigate their biogas formation potential according to the guideline VDI 4630 (2006). The two-phase Gompertz model was used to fit the experimental data (Table 4.4). All bioreactors were agitated.

The biogas formation curves of the substrates are represented in Figure 4.7. The error bars represent the standard deviation for the experimental data. The biogas yield did not significantly differ for the substrates in the three clusters – shavings extruded dry, shavings extruded wet, and shavings treated hydrothermally – (Figures 4.7b, 4.7d, and 4.7c, respectively). A test of analysis of variance (ANOVA) showed no statistically significant difference after the eighth day of anaerobic digestion for all clusters.

The shape of the biogas formation curve for the untreated chromium shavings (Figure 4.7a) indicates a retarded degradation. Agustini et al. (2015) also showed a similar degradation behaviour in batch trials when studying biogas production with chromium shavings. This is the case if the substrate degrades with difficulty (VDI 4630, 2006).

For all substrates tested, R^2 is higher than 0.99 indicating that the experimental data fitted well to the two-phase Gompertz model. Substrates had a one-phase biogas production curve, which can be seen in the two-phase Grompertz model with high values of λ_2 or A_2 of almost 0 L kg^{-1}. The reactors with shavings extruded dry and wet (Figures 4.7b and 4.7d) had very similar behaviours. Compared to untreated chromium shavings (Table 4.4), the lag-phase of the biogas production was reduced to two days due to extrusion pre-treatment. The trials with shavings treated hydrothermally (Figure 4.7c) showed even better results starting biogas production between four and five days before that of the trial with untreated chromium shavings. For the shavings treated hydrothermally at 170 °C, production was observed almost immediately after starting the anaero-

bic reactor, a lag-phase similar to the value of gelatin. The degradation behaviour of the pre-treated shavings rather resembled that of gelatin than that of untreated chromium shavings. Therefore, the more complex the structure of collagen, the later biogas production starts. As the untreated chromium shavings did not have their collagen structure denatured by pre-treatment, the anaerobic sludge needs a longer period of time to hydrolyze the substrate before initiating any biogas production.

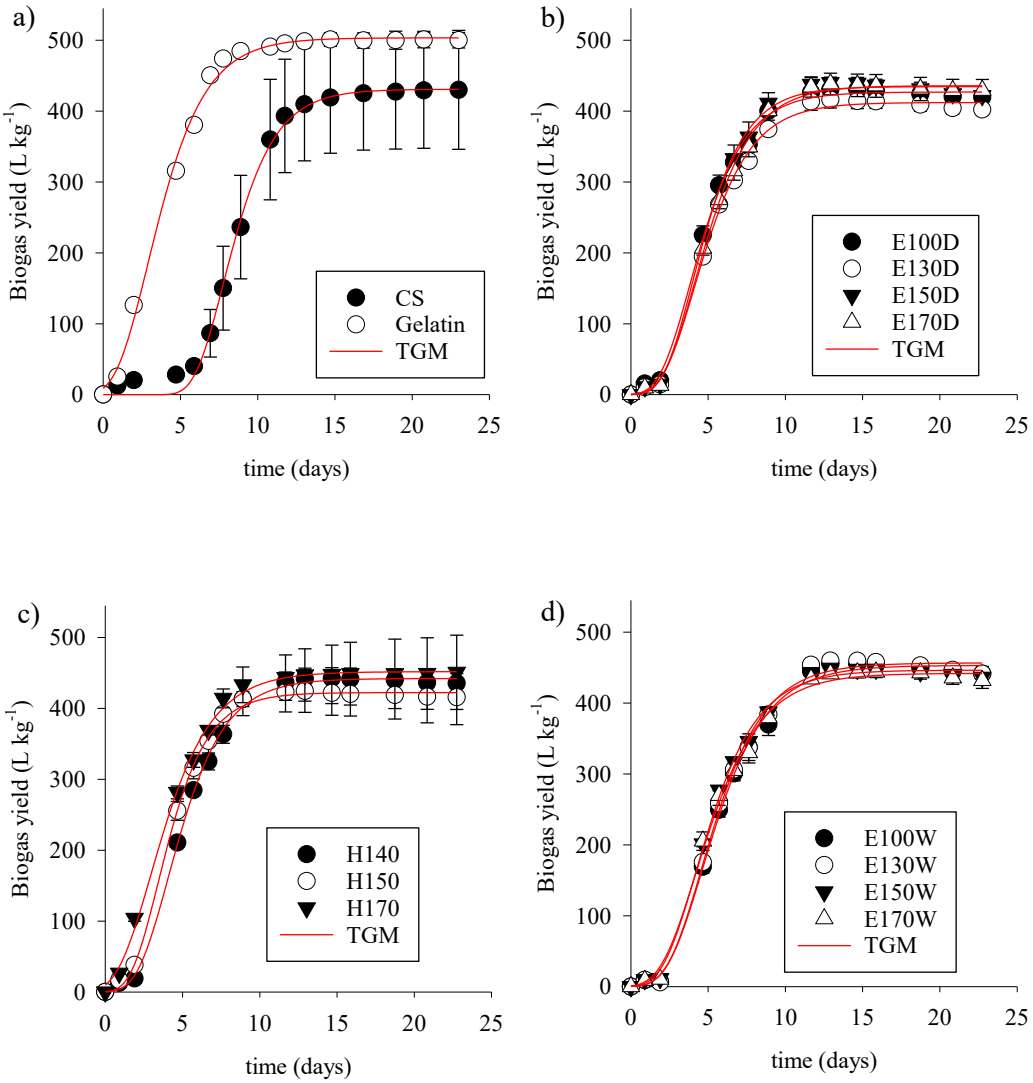

Fig. 4.7: *Cumulative biogas production and two-phase Gompertz simulation (red line) for the untreated shavings and gelatin (a), shavings extruded dry (b), shavings treated hydrothermally (c), and shavings extruded wet (d) in agitated bioreactors.*

The maximum biogas production rate could not be increased by pre-treatments. The results for the pre-treated shavings are between 69.8 and 87.4 L kg^{-1} d^{-1}, while the untreated chromium shavings show a value of 84.5 L kg^{-1} d^{-1}. All substrate effectively hydrolyzed in the hydrolysis phase or by pre-treatment, can be processed at a similar rate in the subsequent stages of anaerobic digestion.

Tab. 4.4: *Parameters of biogas production from the two-phase Gompertz equation.*

	λ_1 (days)	μ_1 (L kg^{-1} d^{-1})	A_1 (L kg^{-1})	λ_2 (days)	μ_2 (L kg^{-1} d^{-1})	A_2 (L kg^{-1})	R^2
Gelatin	0.8	89.0	503.4	∞	-	-	0.9962
CS	5.9	84.5	430.9	-	-	0.0	0.9966
Shavings extruded wet							
	λ_1 (days)	μ_1 (L kg^{-1} d^{-1})	A_1 (L kg^{-1})	λ_2 (days)	μ_2 (L kg^{-1} d^{-1})	A_2 (L kg^{-1})	R^2
E100W	2.3	72.4	453.2	∞	-	0.0	0.9973
E130W	2.3	74.6	456.7	-	-	0.0	0.9966
E150W	2.0	74.6	446.4	∞	-	0.0	0.9975
E170W	1.9	69.8	442.0	∞	-	0.0	0.9964
Shavings extruded dry							
	λ_1 (days)	μ_1 (L kg^{-1} d^{-1})	A_1 (L kg^{-1})	λ_2 (days)	μ_2 (L kg^{-1} d^{-1})	A_2 (L kg^{-1})	R^2
E100D	1.9	78.8	427.0	-	-	0.0	0.9978
E130D	2.0	73.2	412.2	-	-	0.0	0.9976
E150D	2.1	83.8	434.6	-	-	0.0	0.9977
E170D	2.1	77.4	435.9	-	-	0.0	0.9982
Shavings treated hydrothermally							
	λ_1 (days)	μ_1 (L kg^{-1} d^{-1})	A_1 (L kg^{-1})	λ_2 (days)	μ_2 (L kg^{-1} d^{-1})	A_2 (L kg^{-1})	R^2
H140	2.1	79.8	442.2	-	-	0.0	0.9987
H150	1.7	87.4	422.4	-	-	0.0	0.9988
H170	0.8	75.4	451.9	∞	-	0.0	0.9975

The biogas formation potential of all pre-treated shavings was very similar, between 412 and 457 L kg^{-1} (Table 4.4). The trial using shavings extruded wet at 130 °C as substrate reached the highest biogas formation potential, 457 L kg^{-1}. Comparing this with the results of other authors an improvement in the biogas yield can be found. For instance, Priebe et al. (2016) reached a biogas yield of

162.16 L kg^{-1} testing the degradation of chromium-tanned leather shavings in different biotas and Agustini (2018) found a biogas cumulative production of 29.91 L kg^{-1} studying the degradation of chromium-tanned leather shavings using sludge of wastewater treatment as inoculum.

However, the biogas formation of the pre-treated substrates did not reach the theoretical biogas yield of 796 L kg^{-1} calculated with the basic elementary formula of bovine hide collagen and Equation 2.6. Hides are heterotypic fibrils, which mainly contain collagen type I and a significant amount of type III collagen, typically about 20% by mass (Wess, 2008). Knowing this and with data from the UniProt database (The UniProt Consortium) it is possible to obtain the basic elementary formula for bovine hide collagen necessary and calculate the theoretical biogas yield. The trials using pre-treated chromium shavings as substrate reached 52% to 58% of the maximum conversion capacity for the substrate. A conversion rate between 50 and 70% is expected for proteins (VDI 4630, 2006).

Surprisingly, the biogas formation potential of the untreated chromium shavings was similar to the values found for the pre-treated shavings. Considering the values found in the enzyme assays with trypsin and collagenase, the chromium shavings were expected to be less degraded and to generate a considerable amount of biogas less than the pre-treated shavings in biogas trials. The degradation of collagen-based materials in anaerobic digesters appears to be a complex process. If the substrate is not denatured in advance, hydrolysis probably involves several enzymes working simultaneously to degrade the material which requires some days causing a lag-phase. To confirm this and determine the involved enzymes further research is needed.

Comparing these results with the previous section (Table 4.2), one can see that the same substrates with the same agitation conditions obtained higher biogas formation potentials in the present section. The difference is caused by the different substrate to inoculum ratio utilized in the reactor flasks. Better results are achieved using a smaller amount of sample. A reason can be that this smaller

quantity favours the mass transfer inside the reactors and hence the contact between substrate and anaerobic bacteria, while a larger load results in an overload of the reactor and inhibits the anaerobic bacteria.

The degradation degree of collagen was estimated by characterization of the biomass collected at the end of the digestion from each reactor. Table 4.5 shows the characterization of the final biomass and the degradation degree of collagen or hydroxyproline for each substrate tested.

Tab. 4.5: *Biomass characterization after digestion and degradation degree of collagen for bioreactors testing different pre-treatments.*

	Hydroxyproline (%)*	**Degradation degree (%)**	**Chromium (%)****
Gelatin	0.04 ± 0.01	98.8	**-**
	Collagen (%)*	**Degradation degree (%)**	**Chromium (%)****
CS	4.7 ± 0.7	77.2	0.2 ± 0.1
Shavings extruded wet			
E100W	0.3 ± 0.0	98.8	0.8 ± 0.0
E130W	0.3 ± 0.1	99.0	0.5 ± 0.0
E150W	0.2 ± 0.1	99.3	0.6 ± 0.0
E170W	0.2 ± 0.0	99.2	0.4 ± 0.0
Shavings extruded dry			
E100D	0.3 ± 0.0	99.1	1.3 ± 0.1
E130D	0.3 ± 0.0	99.1	1.1 ± 0.1
E150D	0.3 ± 0.1	99.0	0.9 ± 0.0
E170D	0.4 ± 0.1	98.5	0.8 ± 0.1
Shavings treated hydrothermally			
H140	0.4 ± 0.1	98.5	0.6 ± 0.1
H150	0.5 ± 0.1	98.2	0.9 ± 0.1
H170	0.6 ± 0.0	97.8	0.5 ± 0.1

*Dry basis; mean ± standard deviation, n = 3

**Dry basis; mean ± standard deviation, n = 2, measured as chromium oxide

During anaerobic digestion of untreated chromium shavings, the collagen content only partly decreased while for the pre-treated shavings it was reduced

almost to zero. The trials with pre-treated shavings showed a degradation degree above 97% in every case, a value similar to the degradation degree of hydroxyproline in the trial using gelatin. There is a clear difference between trials with pre-treated and untreated shavings which showed a collagen degradation of 77.2% only. Again chromium shavings have proven to be a complex substrate that can be pre-treated to assure high decomposition. However, surprisingly the trials with pre-treated shavings did not reach the same biogas formation potential as gelatin.

The untreated chromium shavings showed the same biogas formation potential as the pre-treated shavings, but they had a longer lag-phase and needed a longer period of time to be degraded. Gelatin was more easily degradable and digestion of the pre-treated shavings was not able to reach its biogas formation potential. If the chromium shavings are denatured by extrusion pre-treatment, the collagen chains are broken down and lose their stability but they are still mainly arranged in long chains. This stiff arrangement also has to be broken down and its degradation is difficult (Suzuki et al., 2006). The effort necessary to degrade the gelatin is much lower because hydrolysation of the main chains has already been achieved by the manufacturing process of gelatin. Therefore, microorganisms will need much more energy to degrade pre-treated chromium shavings than gelatin.

In addition, degradation of gelatin did not reach the theoretical biogas yield of 796 L kg^{-1} calculated with the basic elementary formula of bovine hide collagen and Equation 2.6. Gelatin reached 63.2% of the maximum conversion capacity for the substrate. The value for the biogas formation potential of gelatin is also lower than that found for casein and blood as protein-rich substrates, 775 and 824 L kg^{-1}, respectively (Kovács et al., 2013), meaning that collagen-based materials such as chromium leather waste are expected to yield less biogas compared to other proteinaceous materials. An explanation for this could be that the collagen chain itself is also a substrate which needs much effort to be degraded.

Trials using pre-treated shavings reached low collagen content results within the range of that achieved by degradation of gelatin. This indicates that the chromium content in the biomass of up to 1.3% (measured as chromium oxide)

did not affect degradation of the substrates. Moreover, the pH value of the biomass after digestion was very stable, always between 8 and 8.5, highlighting the stability of the process.

The pre-treatments were important to partially replace the hydrolysis step of the biogas production, represented by the lag-phase, and assure a reduction of the final waste increasing degradation degree of collagen from 77.2% to about 99%. These aspects show that using these pre-treatments would be beneficial to the production of biogas from chromium shavings. As seen in this section and in the previous sections, pre-treated shavings in the same cluster did not significantly differ from each other and showed similar results. Therefore, from the pre-treated shavings, only the shavings extruded dry at 100 °C were selected for further investigations in batch trials.

4.2.1.3 Comparison of different substrate to inoculum ratios

Different substrate to inoculum ratios were tested in batch tests to investigate the effect of the substrate load. Untreated chromium shavings, gelatin (denatured and hydrolyzed collagen), shavings extruded dry at 100 °C, and extruded offcuts were tested as substrates. The two-phase Gompertz model was used to fit the experimental data. Figure 4.8 and Table 4.6 compare the results obtained for these biogas trials. The error bars in Figure 4.8 represent the standard deviation for the experimental data.

A plateau during production, also known as diauxie (two-phase decomposition) was verified for gelatin, shavings extruded dry at 100 °C, and extruded offcuts, with the exception of the 0.5 S/I ratio experiment (Figure 4.8). Some authors support the existence of diauxic growth in anaerobic digestion and different theories are used to explain this. Misi and Forster (2001), Marin et al. (2010), and Walter et al. (2016) attributed this curve to the degradation of low complexity substrates at the beginning of digestion followed by a retarded degradation of more complex substrates afterwards. Ashekuzzaman and Poulsen (2011) indicated adaptation by microorganisms as being the cause of diauxie. Furthermore, this behaviour could be related to the excessive production of volatile fatty acids during anaerobic digestion, which inhibits methane production. Lee et al. (2010) verified a diauxic utilization of acetate to propionate, which results

in a diauxie biogas production curve. Chanakya et al. (2015) related inhibition of the anaerobic bacteria to the accumulation of volatile fatty acids. Kim and Kim (2017) attributed diauxie to the rapid acidification caused by the accumulation of volatile fatty acids.

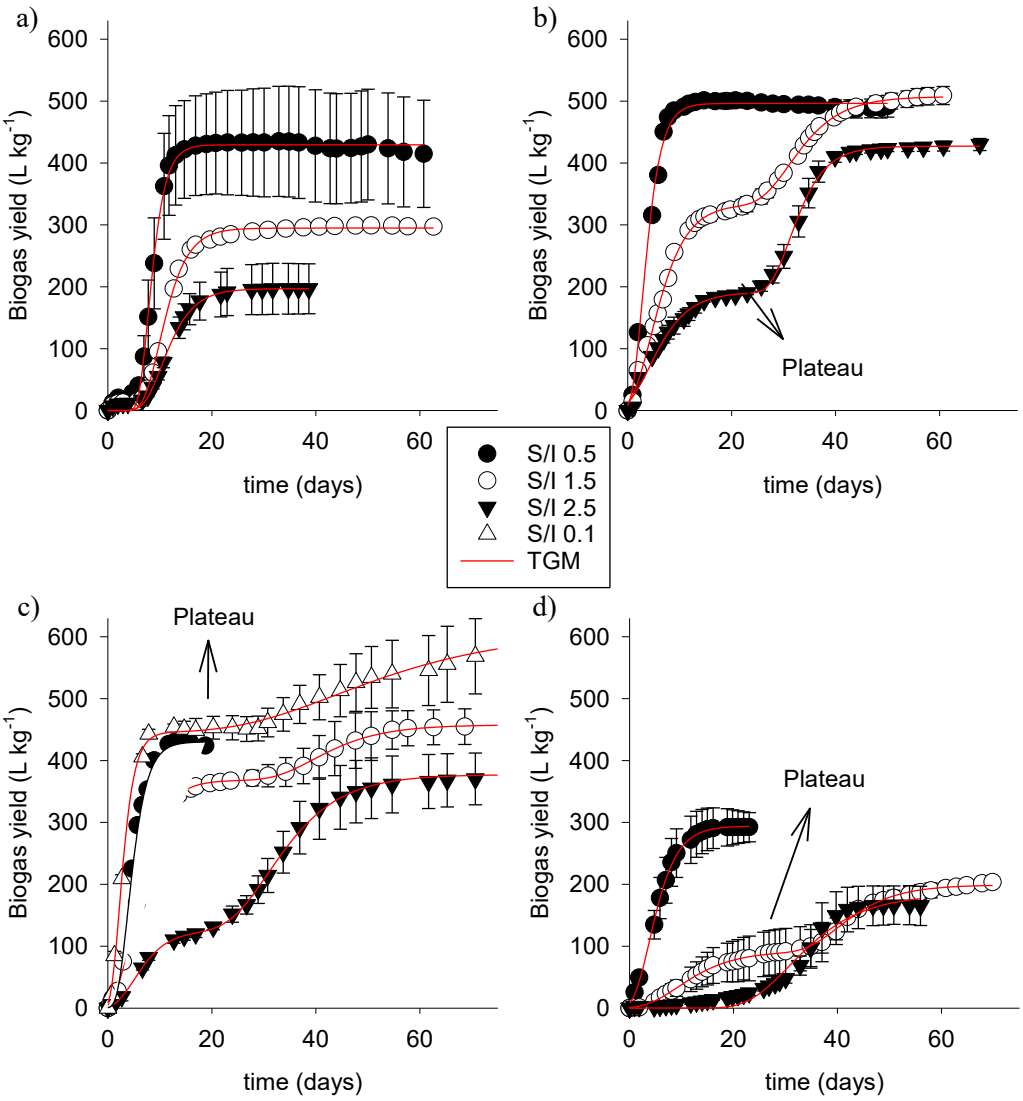

Fig. 4.8: *Cumulative biogas production and two-phase Gompertz simulation (red line) for the untreated chromium shavings (a), gelatin (b), shavings extruded dry at 100 °C (c), and extruded offcuts (d) with different substrate quantities in agitated bioreactors.*

Trials using untreated chromium shavings as substrate did not show diauxie due to the complexity of the material. The substrate is not pre-denatured and

hydrolysis proceeds slowly. For complex substrates hydrolysis is the rate-limiting step in the anaerobic degradation. Therefore, the production of intermediates is slow and they do not accumulate or cause inhibition of microorganisms. A similar case is the anaerobic degradation of cellulose, a complex substrate due to the presence of lignin (Tsavkelova and Netrusov, 2012). The slow hydrolysis of cellulose even allows using higher S/I ratios, which can be found within the range from 1.08 to 4.1 (Liew et al., 2011; Liew et al., 2012; Song et al., 2013).

Tab. 4.6: *Parameters of biogas production from the two-phase Gompertz equation.*

Untreated chromium shavings							
S/I	**λ_1 (d)**	**μ_{m1} (L kg^{-1} d^{-1})**	**A_1 (L kg^{-1})**	**λ_2 (d)**	**μ_{m2} (L kg^{-1} d^{-1})**	**A_1+A_2 (L kg^{-1})**	**R^2**
0.5	5.9	86.5	429.3	∞	-	$A_2 = 0.0$	0.9968
1.5	6.9	35.9	294.9	∞	-	-	0.9979
2.5	6.9	21.3	196.9	-	-	$A_2 = 0.0$	0.9965
Gelatin							
S/I	**λ_1 (d)**	**μ_{m1} (L kg^{-1} d^{-1})**	**A_1 (L kg^{-1})**	**λ_2 (d)**	**μ_{m2} (L kg^{-1} d^{-1})**	**A_1+A_2 (L kg^{-1})**	**R^2**
0.5	0.8	91.0	496.4	-	-	$A_2 = 0.0$	0.9954
1.5	0.8	32.5	333.6	25.6	11.6	507.4	0.9986
2.5	0.0	16.9	191.4	27.4	22.8	427.2	0.9980
Shavings extruded dry at 100 °C							
S/I	**λ_1 (d)**	**μ_{m1} (L kg^{-1} d^{-1})**	**A_1 (L kg^{-1})**	**λ_2 (d)**	**μ_{m2} (L kg^{-1} d^{-1})**	**A_1+A_2 (L kg^{-1})**	**R^2**
0.1	0.6	102.8	445.7	24.9	3.2	608.8	0.9980
0.5	1.8	77.7	430.2	-	-	$A_2 = 0.0$	0.9980
1.5	1.4	41.3	368.4	31.9	4.4	457.6	0.9994
2.5	1.4	12.8	125.5	23.2	12.1	377.3	0.9990
Extruded offcuts							
S/I	**λ_1 (d)**	**μ_{m1} (L kg^{-1} d^{-1})**	**A_1 (L kg^{-1})**	**λ_2 (d)**	**μ_{m2} (L kg^{-1} d^{-1})**	**A_1+A_2 (L kg^{-1})**	**R^2**
0.5	0.8	36.3	294.2	-	-	$A_2 = 0.0$	0.9988
1.5	3.2	5.5	92.2	34.0	6.3	199.1	0.9984
2.5	22.8	8.6	184.0	-	-	$A_2 = 0.0$	0.9767

Almost all conditions tested had an R^2 higher than 0.99 (Table 4.6) except the bioreactor using extruded offcuts with an S/I ratio of 2.5. Substrates which did not show diauxie have a two-phase Grompertz equation with high values of λ_2 or A_2 of almost 0 L kg^{-1}. As seen in the previous section, the batches using gelatin as substrate started production earliest, with a lag-phase between 0 and 0.8 days. The trials with extruded shavings showed a small delay in comparison to gelatin, with a lag-phase between 0.6 and 1.8 days. Using untreated chromium shavings, the lag-phase showed values between 5.9 and 6.9 days. If the substrate is highly complex, i.e., non-denatured, the hydrolytic stage limits the rate of degradation and longer lag-phases can be seen. The hydrolysis time varies with the substrate. In case of leather waste it is a matter of days (Deublein and Steinhauser, 2008).

Additionally, the lag-phase for the extruded offcuts was dependent on the S/I ratio. With an S/I ratio of 0.5 the lag-phase was very short. However, when using a higher ratio at least 3.1 days were necessary to start a very weak biogas production. The S/I ratio of 2.5 for this substrate showed the longest lag-phase in this work of 22.8 days. As extruded offcuts are denatured, hydrolysis should occur quickly and the delay is probably not caused by this stage of digestion. A cause could be the inaccessibility of the substrate due to the wet end and finishing process, as seen in the trypsin assays and solubility in water results (Figures 4.3 and 4.5).

The higher the substrate to inoculum ratio the lower the maximum biogas production rate of the first phase. Similarly, the higher the substrate to inoculum ratio the lower the biogas formation potential (Table 4.6). As recommended in the guideline VDI 4630 (2006), a substrate to inoculum ratio equal or lower than 0.5 should be aimed at. This S/I ratio showed a high biogas formation potential and no inhibition. A substrate to inoculum ratio higher than 0.5 caused inhibition of the anaerobic bacteria responsible for degradation of the substrate and transformation of it into biogas. A ratio markedly lower than 0.5 (S/I ratio of 0.1, only tested for shavings extruded dry at 100 °C) showed improvement in the biogas formation potential but high variance in the results. Nevertheless, a ratio lower than 0.5 should also be avoided as higher loads make the industrial process more economically advantageous.

Gelatin showed the highest biogas formation potential of all substrates tested, followed by the extruded shavings and the untreated chromium shavings. The extruded offcuts showed the lowest biogas formation potential. The shavings extruded dry at 100 °C were proven to be completely denatured by DSC measurements. The denaturation process breaks down the hydrogen bonds that stabilize the triple helix but not necessarily tears apart the long collagen chains. Therefore, the remaining structure is still arranged in unorganized long chains which need to be broken down. In the first step of anaerobic digestion, the hydrolysis, microorganisms need energy to degrade these long chains present in the extruded shavings. In contrast, gelatin is almost ready to start the acidogenesis. Even though the extruded offcuts were also completely denatured when they underwent the same pre-treatment at higher temperatures, they showed the lowest biogas formation potential.

With an S/I ratio of 2.5 (substrate quantity overload), untreated chromium shavings and gelatin had similar biogas yields during the first phase of digestion (A_1), almost 200 L kg^{-1}. However, in the second phase of digestion gelatin was able to produce much more biogas generating a very pronounced diauxie. The trials using extruded shavings as substrate also showed diauxie but reached lower biogas yield values, about 12% less than gelatin. Trials using untreated chromium shavings did not restart the biogas production because intermediate products are produced slowly during hydrolysis and do not overload the next steps in the anaerobic degradation. The extruded offcuts were degraded with difficulty and showed a retarded biogas formation curve (VDI 4630, 2006).

Similarly, for trials with an S/I ratio of 1.5, pre-treated substrates (extruded shavings and offcuts) and gelatin presented diauxie and untreated chromium shavings did not restart the biogas production. Denatured substrates are quickly hydrolyzed and overload the next steps of the anaerobic digestion at this S/I ratio. The diauxie behaviour was more pronounced for gelatin than for extruded shavings. Trials using extruded offcuts as substrate showed diauxie reaching a biogas formation potential of 199 L kg^{-1} and trials using untreated chromium shavings did not show diauxie.

For an S/I ratio of 0.5, no diauxie curve was formed. The biogas formation potential of the substrates could be reached very early in the trials, indicating that all the potential of the inoculum to produce biogas from the substrate was used. The same occurred with the untreated chromium shavings after a lag-phase of six days at the beginning of anaerobic digestion.

Table 4.7 shows the results of characterization of the final biomass and the degradation degree of collagen for each S/I ratio. The reactors loaded with untreated chromium shavings were unable to reach a complete degradation of collagen. A clear relation to the S/I ratio can be seen. A higher ratio results in a higher final collagen and organic matter content and a lower degradation degree of collagen, indicating that part of the substrate remains unprocessed in the biomass. Degradation of collagen was almost complete for all ratios tested when extruded shavings were used. These reactors showed a degradation degree of collagen similar to the degradation degree of hydroxyproline of the gelatin trials. Furthermore, their final organic matter contents were lower than those for the untreated chromium shavings. Extruded offcuts showed a degradation degree of collagen between 90% and 96%, values higher than those found for the untreated chromium shavings. However, a comparison of these values is difficult because extruded offcuts showed a lower value of collagen content, about 50% (Table 4.1). Instead it is more reasonable to compare collagen and organic matter content values in the final biomass of the reactors. The collagen content measured in the final biomass of the reactor with extruded offcuts as substrate was low, lower than that for untreated chromium shavings. However, the organic matter content in this biomass was high, similar to the final biomass of the reactor with untreated chromium shavings.

Trials with untreated chromium shavings revealed a lower degradation of collagen performance compared to the extruded shavings in all S/I ratios. The trials with untreated chromium shavings also revealed lower values for the biogas formation potential than the trials with pre-treated shavings, except for an S/I ratio of 0.5 when values were very similar. The pre-treatment was important to increase degradation of collagen. As previously seen, trials using extruded shavings as substrate did not reach the same biogas formation potential as gelatin because extruded shavings are still arranged in long chains. Consequently,

more energy is necessary for the degradation of extruded shavings than that of gelatin.

Tab. 4.7: *Biomass characterization after digestion and degradation degree of collagen for bioreactors testing different substrate to inoculum ratios.*

Untreated chromium shavings				
S/I	**Organic matter (%)***	**Collagen (%)****	**Degradation degree (%)**	**Chromium (%)*,*****
0.5	47.2 ± 0.1	4.7 ± 0.7	77.2	0.2 ± 0.1
1.5	56.0 ± 0.1	15.6 ± 1.3	69.5	1.7 ± 0.0
2.5	64.0 ± 0.1	32.1 ± 1.3	44.8	2.2 ± 0.0
Gelatin				
S/I	**Organic matter (%)***	**Hydroxypro-line (%)****	**Degradation degree (%)**	**Chromium (%)*,*****
0.5	46.0 ± 1.1	0.04 ± 0.01	98.8	-
1.5	48.8 ± 0.2	0.05 ± 0.01	99.6	-
2.5	49.1 ± 0.4	0.06 ± 0.00	99.6	-
Shavings extruded dry at 100 °C				
S/I	**Organic matter (%)***	**Collagen (%)****	**Degradation degree (%)**	**Chromium (%)*,*****
0.1	43.6 ± 0.2	0.0 ± 0.0	100	0.1 ± 0.0
0.5	45.8 ± 0.1	0.3 ± 0.0	99.1	1.3 ± 0.1
1.5	44.5 ± 0.2	0.3 ± 0.1	99.5	2.0 ± 0.2
2.5	51.3 ± 0.6	0.0 ± 0.0	100	3.6 ± 0.1
Extruded offcuts				
S/I	**Organic matter (%)***	**Collagen (%)****	**Degradation degree (%)**	**Chromium (%)*,*****
0.5	51.9 ± 0.2	1.9 ± 1.0	95.7	-
1.5	59.1 ± 0.6	3.3 ± 0.9	89.9	-
2.5	63.5 ± 0.1	2.0 ± 0.2	95.7	-

*Dry basis; mean ± standard deviation, n = 2
**Dry basis; mean ± standard deviation, n = 3
***Measured as chromium oxide
Source: Gomes et al., 2019a.

Results indicate that extruded offcuts are much more complex than untreated chromium shavings. As seen before in the degradation by trypsin, the wet end

and finishing process in the leather industry made the material even less inaccessible for the production of biogas than untreated chromium shavings. The organic matter content, which comprises not only collagen but also dyes, retanning agents, pigments, and other polymers (Covington, 2009), was very high. The structure of the leather offcuts can be denatured by extrusion pre-treatment, and detectable collagen in the final biomass is almost eliminated, but these high quantities of organic matter remain unprocessed and the biogas formation potential is the lowest among the substrates tested.

The chromium content in the collected biomass samples reached values of up to 3.6% and degradation of collagen was not affected indicating that chromium at this concentration does not affect the bacteria in the anaerobic digestion.

The effective use of chromium shavings to produce biogas depends on many parameters. One of the most important parameters is the direct comparison of the biogas formation potential for different substrates. Table 4.8 shows the biogas formation potential (A) of several substrates used in biogas plants. The biogas formation potential values were calculated from literature data (FNR, 2012) in order to compare the values to the biogas formation potential found for pre-treated chromium shavings in this work.

On average, trials using pre-treated chromium shavings with an S/I ratio of 0.5 showed a biogas formation potential of 441 L kg^{-1}. This value is comparable with those found for manure, which represented 44.5% (by weight) of the substrate input in German biogas plants in 2016 (DBFZ Betreiberbefragung Biogas, 2017). Compared with energy crops, pre-treated chromium shavings showed a lower biogas formation, however, tannery waste has other advantages. The substrate costs can account for up to 50% of the total costs in a biogas plant operated mainly using energy crops as substrate (FNR, 2012). The use of waste to produce biogas does not entail production costs and reduces disposal of waste. Furthermore, the production of energy crops is associated with other problems, such as competition with the food production, land use, and use of monocultures.

Tab. 4.8: *Biogas formation potential of different substrates.*

Manure		**Energy crops**		**Substrates from processing industry**	
Substrate	**A (L kg^{-1})***	**Substrate**	**A (L kg^{-1})***	**Substrate**	**A (L kg^{-1})***
Cattle slurry	313	Maize silage	638	Spent grains	684
Pig slurry	583	Whole-crop cereal silage	606	Cereal vinasse	691
Cattle dung	400	Green rye silage	667	Potato vinasse	667
Poultry manure	467	Cereal grains	735	Fruit pomace	632
Horse manure	300	Grass silage	571	Rapeseed cake	825
Prunings and grass clippings		Sugar beet	628	Potato pulp	684
		Fodder beet	625	Potato juice	1962
Substrate	**A (L kg^{-1})***	Sunflower silage	533	Pressed sugar beet pulp	298
Prunings and clippings	1667	Sudan grass	521	Molasses	421
		Sweet sorghum	539	Apple pomace	481
		Green ryeb	591	Grape pomace	680

*average values, kg of organic dry matter
Source: FNR, 2012.

The substrates from the processing industry show a wide range of biogas formation potential. Their use in biogas plants is not very attractive, they represented only 2.4% (by weight) of the substrate input in German biogas plants in 2016 (DBFZ Betreiberbefragung Biogas, 2017). The reasons vary with the substrate. For instance, brewer's grains and sugar beet are also used in the food industry and as animal feed, and vinasse has a low dry matter content and in some cases is not worth transporting (FNR, 2012). Prunings and grass clippings from the maintenance of parks and green verges show a biogas formation po-

tential considerably higher than that of pre-treated chromium shavings, however their seasonal and disperse production makes their use in biogas plants difficult and they are normally composted (FNR, 2012).

The S/I ratio of 0.5 showed the best results amongst the ratios studied for all the substrates studied (pre-treated or not) and should be aimed at when using these collagen-based materials as substrate for the production of biogas. The substrates studied showed a high biogas formation potential and no diauxie inhibition in the anaerobic digestion using this S/I ratio. Furthermore, the pre-treated chromium shavings reveal a biogas formation potential comparable to that of other substrates commonly used. Their advantages are the availability in large quantities, no costs, and no significant use as a by-product. Their use as a substrate for biogas production would even reduce costs with disposal of waste in tanneries. A disadvantage of their use as substrate to produce biogas is the content of chromium in this material because it complicates the use of the final biomass as a fertilizer. Extraction and recycling of chromium from the final biomass produced in the reactors must be further studied.

4.2.1.4 Diauxie investigation

Hitherto, the diauxie behaviour in anaerobic digestion has not been completely clarified. Following the theory that inhibition is caused by accumulation of intermediate products, supported by Chanakya et al. (2015) and Kim and Kim (2017), the volatile fatty acids produced along diauxie were measured. This would mean that there is an imbalance between hydrolysis and methanogenesis. As the substrate is denatured, it can be quickly hydrolyzed to produce intermediate products. However, methanogenesis is not accelerated and these intermediate products accumulate thus inhibiting methanogenic archaea. This only occurs for an S/I ratio higher than 0.5 and denatured substrates (Gomes et al. 2019a).

To investigate this, trials of the previous section in which diauxie was verified were repeated. Bioreactors for gelatin, shavings extruded dry at 100 °C, and extruded offcuts (S/I ratio of 1.5) were started seven times in triplicate simultaneously. Every triplicate was terminated at different times in the anaerobic di-

gestion to collect a sample of biomass for analytical investigations. The concentrations of volatile fatty acids in each batch of the seven samples were determined by high-performance liquid chromatography. Diauxic growth curve, sampling time, and volatile fatty acid concentration of the samples are shown in Figure 4.9. The error bars represent the standard deviation for the experimental data.

The pH values were stable during anaerobic digestion, increasing only slowly from 7.2 to 8.4, and causing no problems to the process. Even though the volatile fatty acid concentration increased, the pH value was stable. Ashekuzzaman and Poulsen (2011), and Misi and Forster (2001) also verified pH values slightly above neutral in anaerobic digestion batches with diauxic growth. However, the volatile fatty acid concentration during the batches was not measured.

In their paper, Ramsay and Pullammanappallil (2001) reviewed the products resulting from fermentation of amino acids using the Stickland reaction. Based on that, it was possible to define the products mainly expected for fermentation of the amino acids of bovine hide collagen. The most relevant volatile fatty acids produced in the three reactors were acetic acid, propionic acid, and isovaleric acid, which is consistent with the amino acid composition of the substrate. The most relevant amino acids present in these collagen chains are glycine, which is fermented to acetic acid, and proline, which is fermented to acetic acid, propionic acid, and valeric acid. Most of the other amino acids present in the collagen chains are fermented to acetic acid but some are also fermented to propionic acid and butyric acid (Ramsay and Pullammanappallil, 2001), which is also present in the reactors in low concentrations.

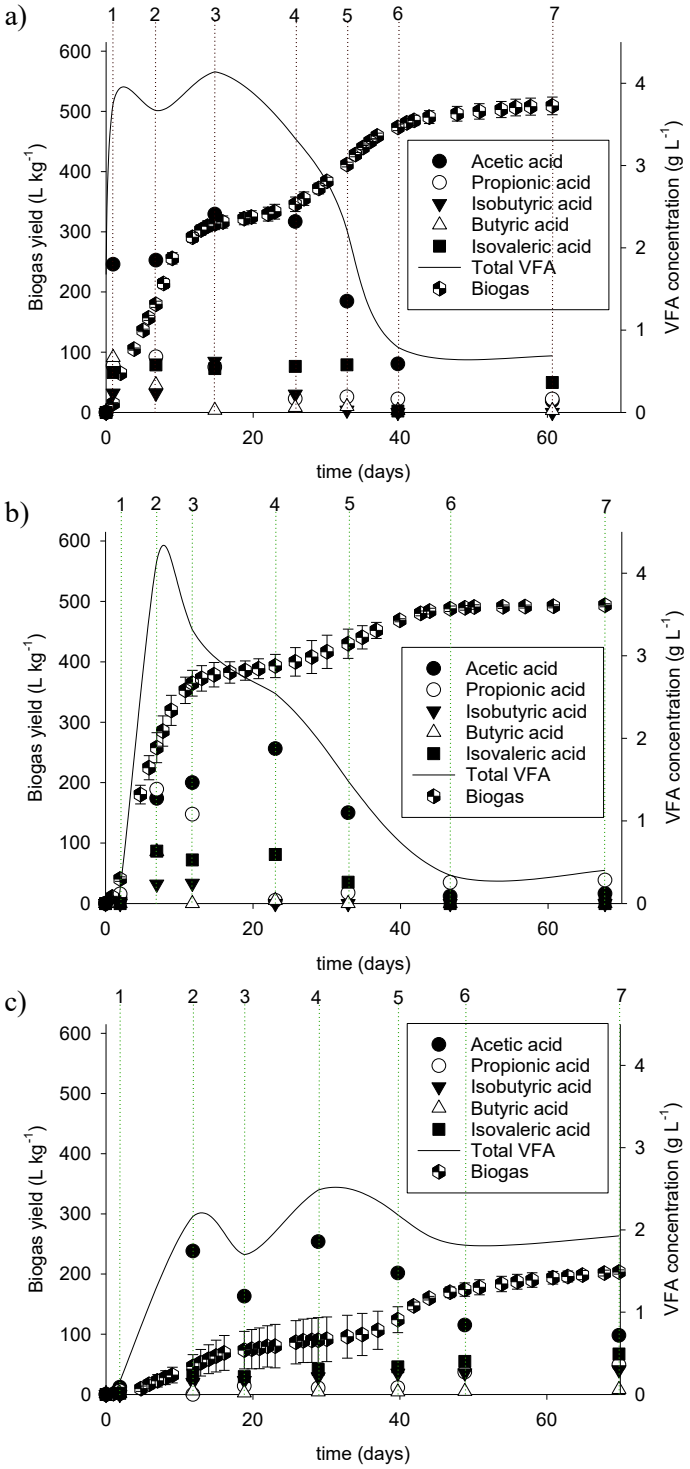

Fig. 4.9: *Reproduction of the biogas yield, sampling time, and volatile fatty acid (VFA) concentrations present in samples collected for gelatin (a), shavings extruded dry at 100 °C (b), and extruded offcuts (c) in agitated bioreactors (source: Gomes et al., 2019a).*

For the extruded shavings (Figure 4.9b), biogas production was suppressed in case the volatile fatty acid concentration was high. Chanakya et al. (2015) observed the same behaviour. This means that during acidogenesis a large amount of these acids is produced, which are intermediate products for generating biogas. However, the bacteria are not able to process all of the produced acids accumulating. As these acids are consumed and transformed into substrates to produce methane during acetogenesis, their concentration is reduced and methane production resumed. Lee et al. (2010) also measured the volatile fatty acid concentration during anaerobic digestion and the same behaviour was verified. The volatile fatty acids were mainly composed of acetic and propionic acids. Propionic acid showed a concentration higher than 1.0 g L^{-1} in the second and third sample, a concentration known to cause inhibition (Kaiser et al., 2008).

Gelatin (Figure 4.9a) showed the same behaviour as the extruded shavings. However, the volatile fatty acid concentration was already very high in the first biomass sample because gelatin started producing biogas faster than the extruded shavings. The chains of gelatin partly hydrolyzed start production of volatile fatty acid immediately after substrate and inoculum are in contact. The propionic acid concentration was not as high as observed for the trial with extruded shavings, but for the first, second, and third biomass samples collected it was higher than 0.3 g L^{-1}, a concentration discussed to disturb anaerobic digestion (Deublein and Steinhauser, 2008). The isobutyric acid concentration was higher than the inhibition concentration of 0.5 g L^{-1} (Kaiser et al., 2008) in the third biomass sample collected.

The reactor containing the extruded offcuts (Figure 4.9c) showed high volatile fatty acid concentrations (about 2 g L^{-1}) during all digestion stages. The volatile fatty acid results indicate that there could be inhibition by these intermediate products. Furthermore, the highest volatile fatty acid concentration (2.5 g L^{-1}) was verified during the diauxic plateau-phase. Unlike the extruded shavings, this reactor shows a high concentration of volatile fatty acid until the end of digestion. A high propionic acid concentration of 0.4 g L^{-1} was also verified at the end of the process for this reactor. These results could indicate that the production of biogas reaches its end because of inhibition and that, consequently,

part of the available organic matter found in the biomass collected was not transformed into biogas.

The anaerobic digestion of collagen was previously classified as biphasic by Lalitha et al. (1994). In their paper, a trial with substrate overload showed two peaks of biogas production. The first peak was at the beginning of digestion when collagen was being hydrolyzed and acidogenesis started. This peak produced a biogas mainly composed of CO_2. As the system was overloaded, there was a rapid increase in the volatile fatty acid concentration, which is detrimental to the methanogenic population. The second peak corresponded with the beginning of the methanogenesis, when the volatile fatty acid concentration dropped. These results correspond with those represented in this paper.

Diauxie only occurs in trials with substrates already denatured (gelatin, extruded shavings, and extruded offcuts) and with an S/I ratio higher than 0.5, because these substrates are quickly hydrolyzed to form volatile fatty acids during acidogenesis. However, the bacteria are not able to process all of the intermediate products that fast, which means that they start to accumulate and biogas production drops. Production continues when the volatile fatty acid concentration drops. This indicates that when using denatured collagen as substrate, methanogenesis is the rate-limiting step during anaerobic degradation instead hydrolysis.

Accumulation of volatile fatty acid produced during acidogenesis could be the cause of the diauxie behaviour of the biogas production curves. It is still necessary to find out if accumulation of volatile fatty acid is responsible for inhibition of methanogenesis or a consequence of inhibition.

Bovine hide collagen is a nitrogen-rich substrate with a C/N ratio of 3.1, calculated with the basic elementary formula (The UniProt Consortium). Therefore, inhibition by ammonia is possible, which is a product resulting from fermentation of amino acids (Nisman, 1954). The ammonia content in the reactors was monitored during digestion. Figure 4.10 shows the results. The sample point 0 represents the ammonia content of the inoculum.

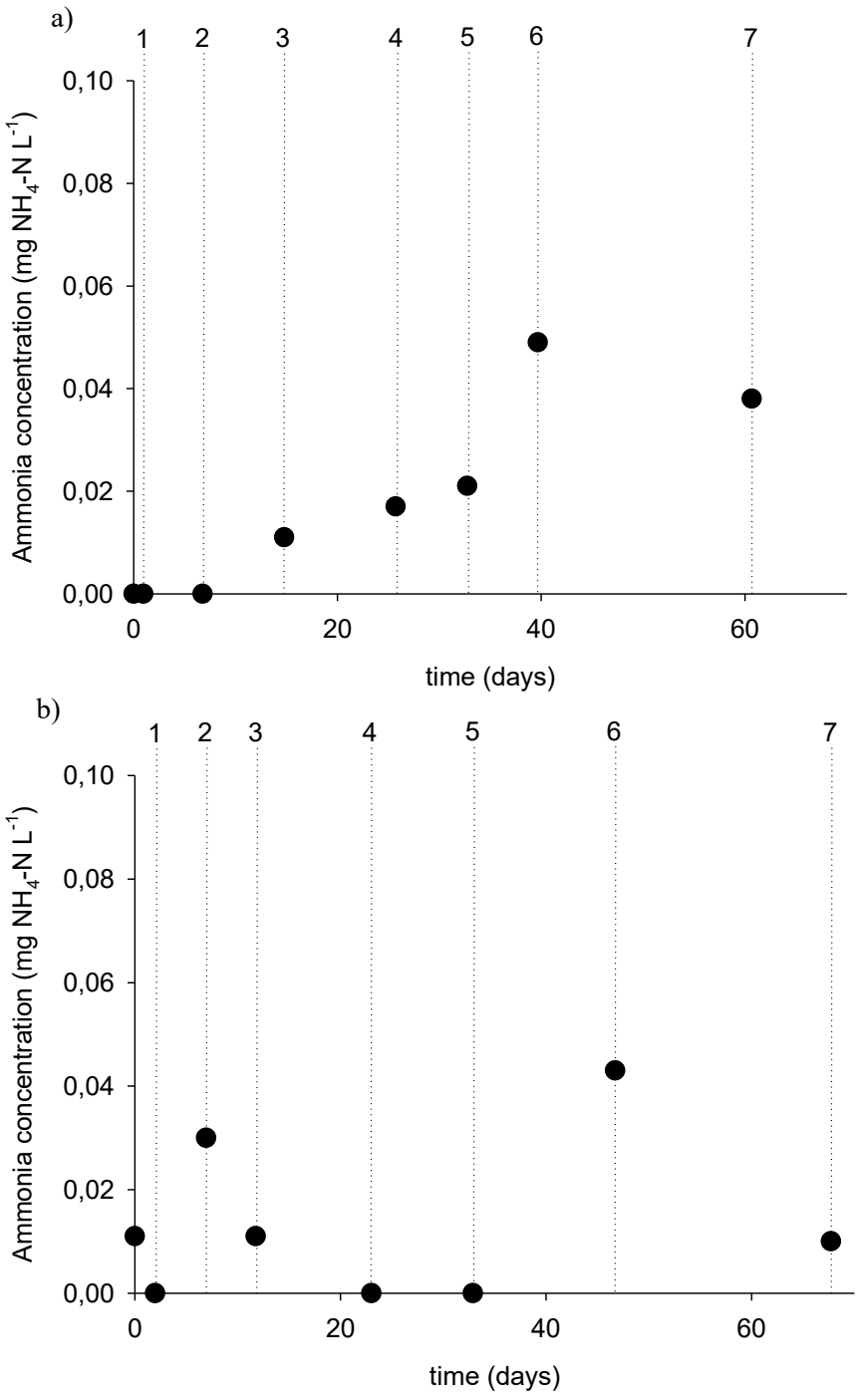

Fig. 4.10: *Concentration of ammonia found in the biomass collected at different reaction times for gelatin (a) and shavings extruded dry at 100 °C (b) in agitated bioreactors.*

For gelatin and extruded shavings, ammonia is present in the biomass in very low concentrations, always below inhibition level, which starts at a concentration of 1.5 to 3.0 g NH_4-N L^{-1} (Drosg et al., 2013), and causes no problem to the anaerobic digestion. Ammonia did not accumulate in the bioreactors probably because of its utilization for generating bacterial biomass. Lalitha et al. (1994) also verified low levels of ammonia during anaerobic digestion of collagen. For the extruded offcuts determination of the ammonia concentration was not possible due to the presence of dyes in the biomass, which interfered the photometric measurement.

Table 4.9 shows the characterization of the biomass sample collected in every sampling point for the three substrates studied. Biomass was analyzed regarding its collagen or hydroxyproline content and organic matter content, and the degradation degree of its collagen or hydroxyproline was estimated.

At the first sampling point the trial using gelatin as substrate already showed a very high degradation degree of hydroxyproline, 97%, while the trial with extruded shavings showed a degradation degree of collagen of about 24% and that with extruded offcuts 8%. Even though extrusion of the collagen structure eases the onset of digestion, a short adaptation period is necessary to start digestion of the previously extruded samples. The short hydrolyzed chains of gelatin can start acidogenesis almost immediately. From sampling point 2 on, the hydroxyproline of gelatin was almost completely degraded showing a degradation degree of about 99% and the degradation degree of the collagen from the extruded shavings increased slowly from 96% to about 99%. For the extruded offcuts, degradation of collagen resulted in a high value (90%) only with regard to the last sample point.

As previously seen for the other batch reactors, degradation of collagen is almost complete at the end of the digestion. The organic matter is also reduced but not completely removed. The organic matter present at the end of digestion, which is not identified as collagen, has to be further investigated. Lalitha et al. (1994) verified a reduction of 85% of collagen content but a decrease of only 65% of organic matter. The volatile fatty acid concentration is very low at the end of digestion and, therefore, could not be the cause of this difference.

Tab. 4.9: *Biomass characterization during digestion regarding its organic and collagen or hydroxyproline content and biogas yield for bioreactors investigating diauxie.*

Gelatin

Sample	Time (days)	Organic matter (%)*	Hydroxyproline (%)**	Degradation degree (%)	Biogas yield (L kg^{-1})**
0	0.0	73.3***	-	-	-
1	0.9	61.2 ± 0.0	0.23 ± 0.02	97.3	65 ± 2
2	6.8	55.5 ± 0.1	0.05 ± 0.00	99.5	168 ± 6
3	14.8	52.3 ± 0.1	0.05 ± 0.01	99.5	317 ± 5
4	25.7	52.1 ± 0.5	0.00 ± 0.01	100.0	334 ± 15
5	32.7	51.0 ± 0.2	0.08 ± 0.01	99.2	391 ± 9
6	39.7	50.9 ± 0.3	0.07 ± 0.01	99.4	489 ± 14
7	60.7	48.8 ± 0.2	0.05 ± 0.01	99.6	509 ± 15

Shavings extruded dry at 100 °C

Sample	Time (days)	Organic matter (%)*	Collagen (%)**	Degradation degree (%)	Biogas yield (L kg^{-1})**
0	0.0	66.9***	31.6***	-	-
1	2.0	66.8 ± 0.2	23.9 ± 1.3	27.6	42 ± 2
2	6.9	55.2 ± 0.0	1.6 ± 0.0	96.3	245 ± 11
3	11.8	52.3 ± 0.1	0.9 ± 0.1	98.0	360 ± 7
4	23.0	51.2 ± 0.2	0.7 ± 0.0	98.5	398 ± 6
5	32.9	50.1 ± 0.5	0.7 ± 0.2	98.6	428 ± 15
6	46.7	47.5 ± 0.3	0.7 ± 0.1	98.7	483 ± 15
7	67.7	46.6 ± 0.1	0.5 ± 0.0	99.1	494 ± 3

Extruded offcuts

Sample	Time (days)	Organic matter (%)*	Collagen (%)**	Degradation degree (%)	Biogas yield (L kg^{-1})**
0	0.0	71.1***	22.2***	-	-
1	1.9	72.8 ± 1.3	20.1 ± 1.3	8.4	6 ± 2
2	11.8	68.1 ± 1.1			60 ± 15
3	18.8	67.5 ± 0.7	12.1 ± 4.9	53.2	113 ± 27
4	28.9	63.7 ± 0.3	12.0 ± 3.0	57.9	120 ± 11
5	39.7	63.0 ± 0.5	15.2 ± 3.3	47.1	124 ± 20
6	48.8	61.5 ± 0.0	9.8 ± 3.9	67.6	192 ± 14
7	69.8	59.1 ± 0.6	3.3 ± 0.9	89.9	203 ± 4

*Dry basis; mean ± standard deviation, n = 2

**Dry basis; mean ± standard deviation, n = 3

***Mean value for all reactors at the start of digestion

As mentioned before, amino acids in anaerobic digesters are mainly fermented in pairs through the Stickland reaction (Schink and Stams, 2013; Ramsay and Pullammanappallil, 2001). The reaction requires one hydrogen donor and one hydrogen acceptor to occur. One third of the amino acid composition in collagen is made up of glycine. The amino acids proline and hydroxyproline are also present in high quantities. These amino acids are known to be hydrogen acceptors, consequently there is a shortage of amino acids in the medium. Specifically, for bovine hide collagen, the shortage is at least 11.5% considering that all other amino acids act as hydrogen donors.

In their paper, Lalitha et al. (1994) also measured collagen through determination of the amino acid hydroxyproline. It is possible that hydroxyproline is fermented and, therefore, cannot be measured while other amino acids remain unprocessed. The presence of unprocessed amino acids could be the cause of the difference between collagen content and organic matter content. Another possibility is the dehydroxylation of hydroxyproline to proline, which would not be detected by the method. A large quantity of other amino acids from the collagen chains could be unprocessed in the biomass but the method is unable to detect them.

The seven biomass samples collected during anaerobic digestion were also analyzed regarding their chemical oxygen demand (COD) as another indicator of organics in the biomass. Results for the three substrates tested are shown in Figure 4.11.

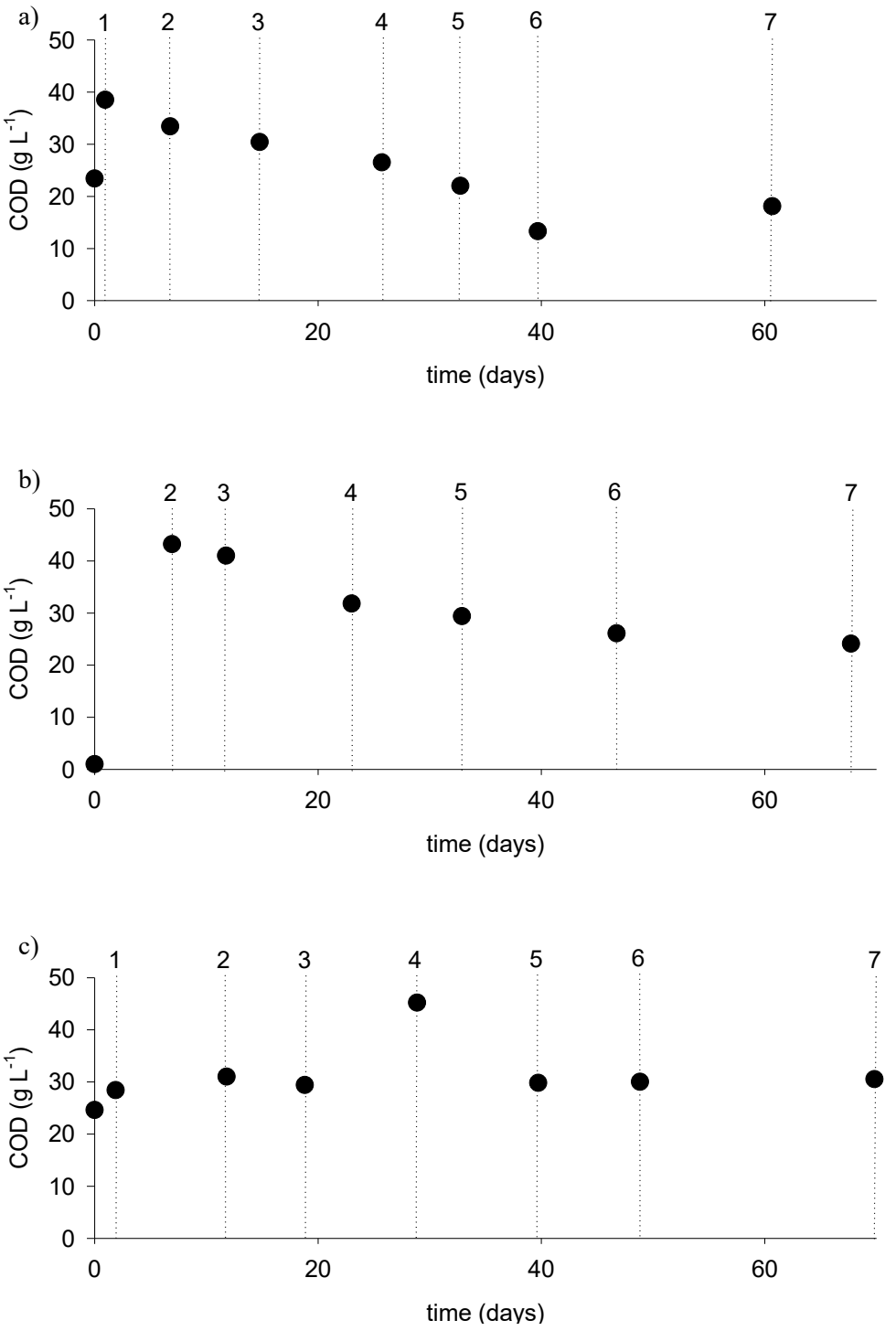

Fig. 4.11: *COD of the biomass samples collected at different reaction times for gelatin (a), shavings extruded dry at 100 °C (b), and extruded offcuts (c) in agitated bioreactors.*

Similarly to the organic matter results, COD values also decreased slowly during digestion, indicating effective destruction of the substrates. An exception were the biomass samples collected from the extruded offcuts reactors. The COD values at the beginning of anaerobic digestion (sample 1) were lower than those for the other two substrates. Presumably this is observed because the substrate is very inhomogeneous. The extruded offcuts were not completely disintegrated in the biomass. Small pieces of the substrate could still be seen. These pieces of substrate were not used for the COD analyses resulting in the lower COD values. However, the biomass of the fourth reactor (sample 4) showed the highest COD value among all reactors studied, indicating that a small piece of undisintegrated substrate was present in the analysed aliquot. COD values were also constant for most of the anaerobic digestion confirming that the organic part of the substrate, which remains unprocessed for the extruded offcuts, was larger.

A high concentration of volatile fatty acid and the excess of propionic acid could cause inhibition of the methanogenic archaea and the diauxic production of biogas in bioreactors with overload of denatured substrate. However, it is also possible that inhibition of the methanogenesis has a different reason and accumulation of volatile fatty acid is a consequence of inhibition rather than its cause.

To evaluate the inhibitory effect of accumulation of volatile fatty acid during anaerobic digestion, bioreactors with gelatin as substrate were injected with different volatile fatty acids. The S/I ratio used, approximately 0.5, is known not to cause diauxie, and the acids were injected when the biogas production was expected to increase. Three different volatile fatty acids were evaluated, acetic acid, propionic acid, and isobutyric acid. The acid concentrations tested involved the inhibitory concentration found in the literature for these acids (Deublein and Steinhauser, 2008; Kaiser et al., 2008), a lower concentration, and a higher concentration. Acids were injected on the second day of digestion. Additionally, the same quantity of acid was injected on the fifth day of digestion. Figure 4.12 shows the biogas production curves obtained from the experiments, the injection time, the injected acid, and its expected concentration in the bioreactor. The error bars represent the standard deviation for the experimental data.

The pH values of the biomass at the end of anaerobic digestion were slightly above neutral (between 7.8 and 7.9), apparently causing no problems to the biogas production process. However, it was not possible to measure the pH value during anaerobic digestion and at the moment of volatile fatty acid injection because there would be ingress of oxygen and this would interrupt the anaerobic digestion. The injection of volatile fatty acid could have a temporary effect on the pH value.

All reactors with injected volatile fatty acids produced more biogas than the reference reactor in which no volatile fatty acid was injected. The higher the volatile fatty acid concentration the higher the biogas formation potential. This contradicts the theory that accumulation of volatile fatty acids inhibits the biogas production forming diauxie. It is rather that the acids, which are described to have an inhibitory effect, are quickly metabolized by the anaerobic bacteria and transformed into biogas without disturbing anaerobic digestion. This leads to the conclusion that the high concentration of volatile fatty acid and the excess of propionic acid verified in the diauxie trials (Figure 4.9) are a consequence of inhibition of the methanogenic archaea rather than its cause. Therefore, accumulation of volatile fatty acid is not the cause of the diauxie curves verified for the substrates studied.

Volatile fatty acids were not detected in the final biomass of the bioreactors. Photometric analyses were performed using a Spectroquant cell test which revealed an acid concentration below the measuring range (< 100 mg L^{-1}). These results indicate that the injected acid was completely transformed into biogas, and they also support the findings previously obtained by other authors. Breure et al. (1986) also studied the addition of volatile fatty acids in bioreactors using gelatin as substrate and concluded that the volatile fatty acids were not inhibitory to the degradation of gelatin. Franke-Whittle et al. (2014) investigated the effect of different levels of volatile fatty acid in anaerobic digesters on the methanogenic archaea and concluded that a high concentration of these acids had no significant effect on the community of methanogenic archaea.

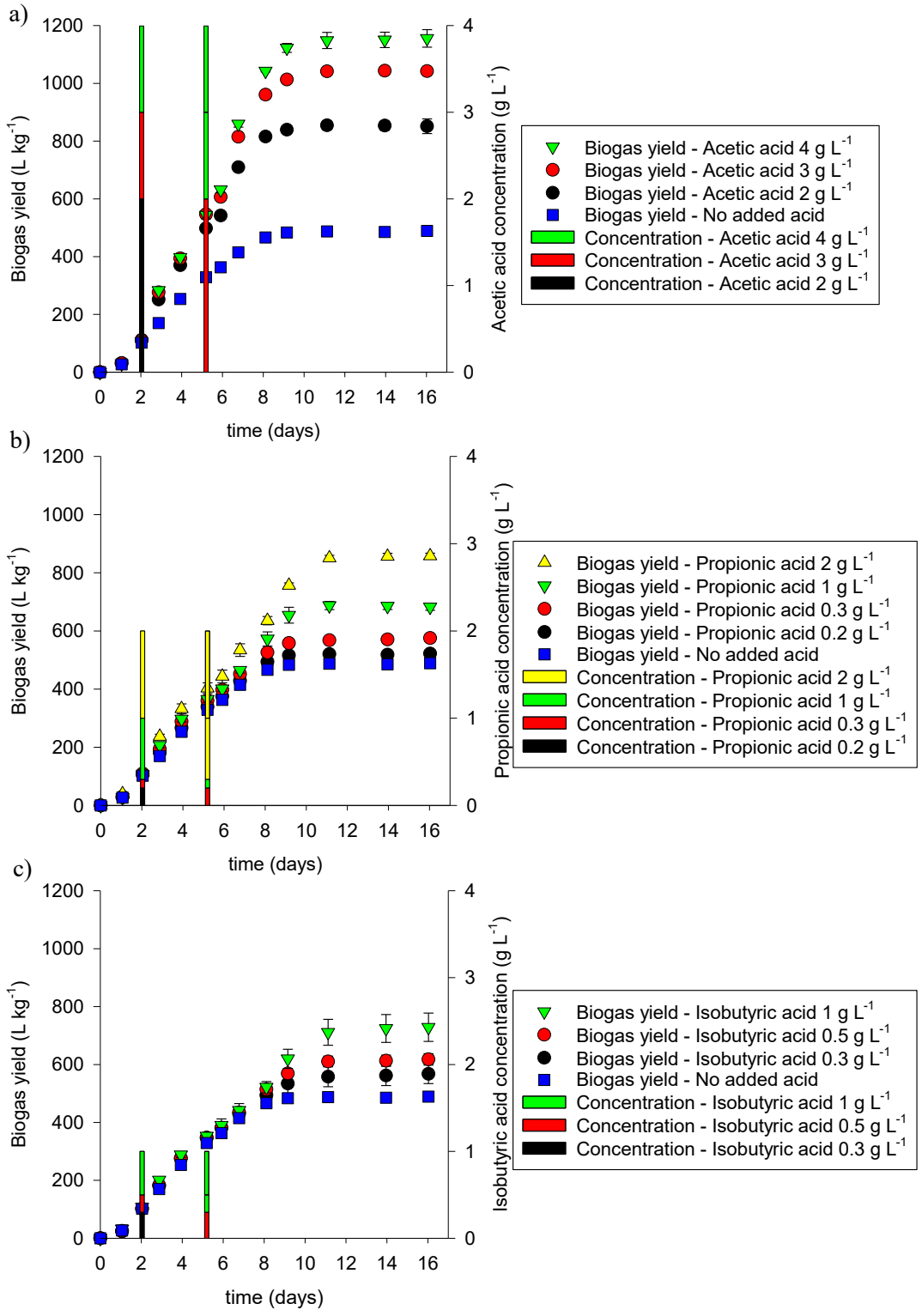

Fig. 4.12: *Cumulative biogas production for the gelatin in the bioreactors with added acetic acid (a), propionic acid (b), and isobutyric acid (c).*

The ammonia content in the reactors was analyzed photometrically in the final biomass using Spectroquant cell tests. Ammonia was below the measuring range (< 0.01 mg L^{-1}) in the final biomass of all bioreactors indicating its use for generating bacterial biomass and probably not causing any inhibition during anaerobic digestion.

Table 4.10 shows the characterization of the final biomass collected at the end of digestion. Biomass was analyzed regarding its hydroxyproline content and organic matter content and the hydroxyproline degradation was estimated.

Tab. 4.10: *Biomass characterization during digestion, degradation degree of hydroxyproline, and biogas yield for bioreactors investigating diauxie.*

Injection of acetic acid				
Acid conc. (g L^{-1})	**Organic matter (%)***	**Hydroxyproline (%)***	**Degradation degree (%)**	**Biogas formation potential (L kg^{-1})**
4	50.7 ± 0.7	0.05 ± 0.01	98.2	1156 ± 30
3	50.1 ± 0.2	0.05 ± 0.00	98.2	1042 ± 8
2	50.4 ± 0.2	0.06 ± 0.00	97.8	852 ± 25
Injection of propionic acid				
Acid conc. (g L^{-1})	**Organic matter (%)***	**Hydroxyproline (%)***	**Degradation degree (%)**	**Biogas formation potential (L kg^{-1})**
2	50.6 ± 0.3	0.05 ± 0.00	98.2	858 ± 10
1	50.4 ± 0.2	0.05 ± 0.01	98.2	683 ± 12
0.3	49.9 ± 0.3	0.06 ± 0.01	98.3	576 ± 8
0.2	49.3 ± 0.3	0.02 ± 0.00	99.6	522 ± 10
Injection of isobutyric acid				
Acid conc. (g L^{-1})	**Organic matter (%)***	**Hydroxyproline (%)***	**Degradation degree (%)**	**Biogas formation potential (L kg^{-1})**
1	49.5 ± 0.0	0.04 ± 0.00	98.7	729 ± 49
0.5	49.6 ± 0.5	0.02 ± 0.01	99.2	618 ± 20
0.3	49.9 ± 0.2	0.03 ± 0.01	99.1	568 ± 34
No added acid				
Acid conc. (g L^{-1})	**Organic matter (%)***	**Hydroxyproline (%)***	**Degradation degree (%)**	**Biogas formation potential (L kg^{-1})**
0	49.8 ± 0.3	0.01 ± 0.00	99.6	489 ± 12

*Dry basis; mean ± standard deviation, n = 2

Even though the biogas formation potential increases substantially for the bioreactors with added acid, results for organic matter, hydroxyproline content, and degradation degree of hydroxyproline show very similar values with regard to the reference trial, with no added acid. Generally, a slight increase in organic matter and hydroxyproline content can be seen for the bioreactors with a high concentration of added acid, which could indicate that anaerobic bacteria would preferably use the injected volatile fatty acid rather than degrade the substrate. However, the increase was very small indicating that the injection of acid did not affect the degradation of gelatin.

Results in this section show that diauxie during anaerobic digestion of collagen-based materials only occurs if the substrates were already denatured with a pretreatment and if there is a substrate overload (S/I ratio higher than 0.5). In this case, the rate-limiting step in the anaerobic degradation is assumed to be methanogenesis rather than hydrolysis. Moreover, it was proved that accumulation of volatile fatty acid is a consequence of inhibition of methanogenesis rather than its cause. The exact inhibitor or inhibitors of the methanogenic archaea remain to be determined.

4.2.1.5 Repeatability of biogas batch trials

To control the variation of the experiments, the same batch trial was performed in two different occasions for comparison. The inoculum used was collected at different days and the gelatin prepared as substrate for the trials was from different batches. These trials can also be seen in previous sections (Sections 4.2.1.3 and 4.2.1.4) and use almost the same S/I ratio. The two-phase Gompertz model was used to fit the experimental data. Figure 4.13 and Table 4.11 compare the results obtained for these biogas trials.

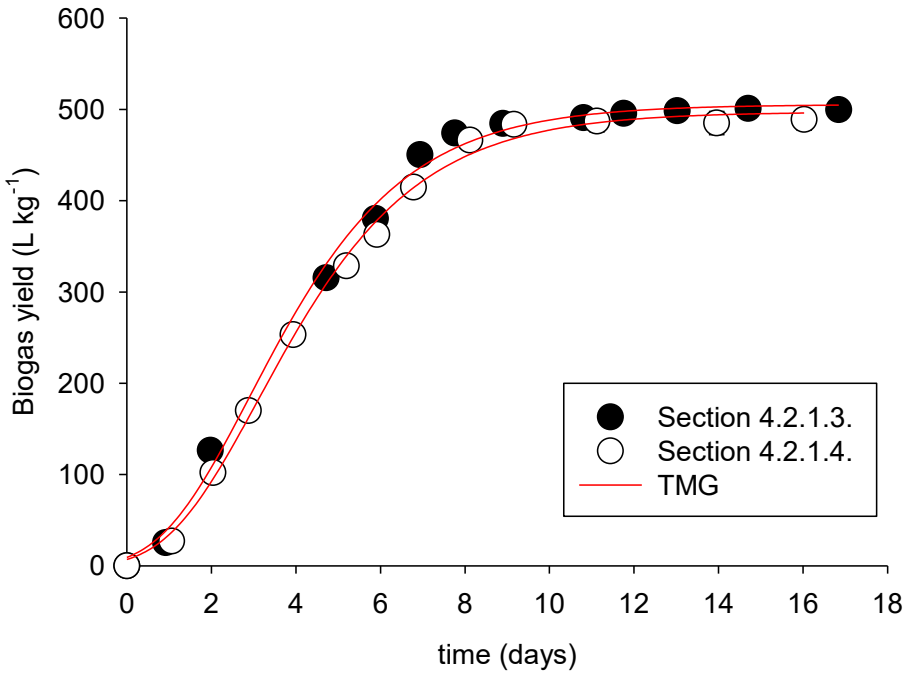

Fig. 4.13: *Cumulative biogas production and two-phase Gompertz simulation (red line) for the gelatin in two different trials with the same conditions.*

Tab. 4.11: *Parameters of biogas production from the two-phase Gompertz equation.*

Section	λ_1 (d)	μ_{m1} (L kg^{-1} d^{-1})	A_1 (L kg^{-1})	λ_2 (d)	μ_{m2} (L kg^{-1} d^{-1})	A_2 (L kg^{-1})	R^2
4.2.1.3	0.8	88.5	505.4	-	-	0.0	0.9960
4.2.1.4	1.0	85.0	497.3	-	-	0.0	0.9969

Both trials had R^2 higher than 0.99 (Table 4.11) and did not show diauxie, which can be seen on the A_2 value of almost 0 L kg^{-1}. Results show that the biogas production curves of the two different batches present very similar behaviour. Furthermore, the parameters from the Gompertz model are very similar. This indicates that the trials can easily be repeated at different occasions and the results obtain from different batches can be compared and trusted.

4.2.2 Pilot scale: continuous tests

The scale-up of the process was necessary to have a better understanding of the method in an industry-like environment. Trials were performed in a continuous

reactor using untreated chromium shavings, shavings extruded dry at 100 °C, and a mixture of equal amounts of shavings treated hydrothermally at 140 °C and 150 °C. The mixture of shavings treated hydrothermally was used due to the large amounts of substrate necessary in continuous trials and because of the similarity between the two substrates. However, samples treated hydrothermally have a high quantity of water, which would excessively increase the working volume of the reactor. Therefore, it was necessary to dry and manually grind the samples before feeding, a process that was not needed for the batch trials with the same substrate.

4.2.2.1 Productivity of the reactors

A low loading rate of 0.5 g kg^{-1} d^{-1} was used to initiate the experiments. Consequently, the loading rate was raised by approximately 0.5 units every time the daily methane production reached a plateau or decreased. Figure 4.14 shows the time plot of digestion for the substrates studied.

Drops in the daily methane production, indicating technical problems, can be seen on the seventh day of digestion for the reactor fed with extruded shavings (Figure 4.14b) and on the 21st day for the reactor fed with shavings treated hydrothermally (Figure 4.14c). The former was caused by an oxygen infiltration on the fifth day due to the rupture of the dip tube used to feed the reactor; the latter was a consequence of an agitation failure, which resulted in uneven heating of the reactor. Even though there is a drop in the methane production, the system recovered its former stability and appeared to function normally after a few days. The reactor fed with shavings treated hydrothermally needed two days only to recover, and the reactor fed with extruded shavings needed five days, that is more time to recover probably because the ingress of oxygen occurred rather early in the digestion process. The daily methane production became very unstable for the extruded shavings when using loading rates higher than 1.4 g kg^{-1} d^{-1} and for the shavings treated hydrothermally for loading rates of 2.0 g kg^{-1} d^{-1}.

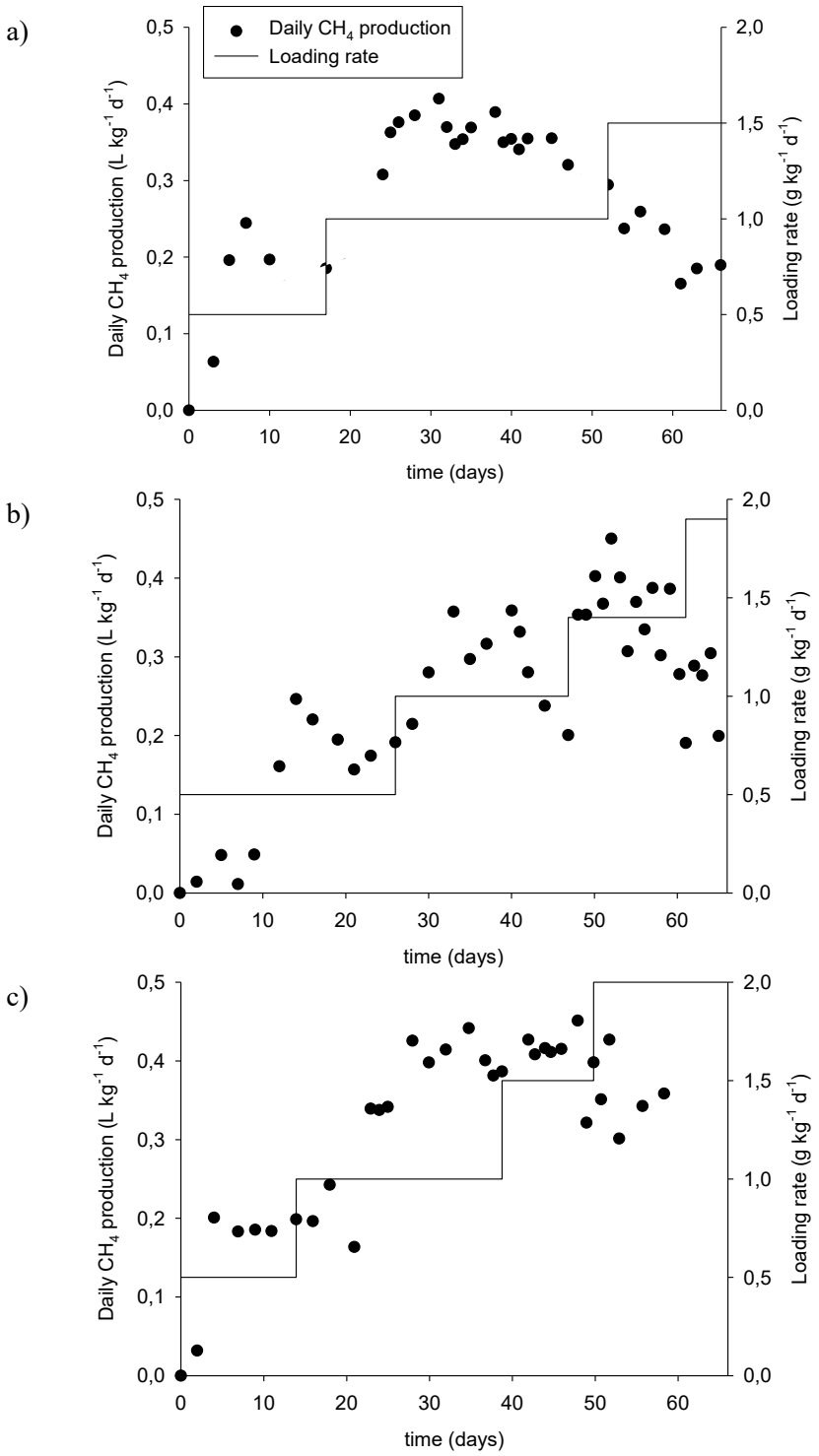

Fig. 4.14: *Time plot of continuous anaerobic digestion of the untreated chromium shavings (a), shavings extruded dry at 100 °C (b), and shavings treated hydrothermally at 140 °C and 150 °C (c) (source: Gomes et al., 2019b).*

The production of a second batch of shavings extruded dry at 100 °C was necessary to feed the reactor starting on the 42nd day. This second batch of the substrate was not completely denatured because it was not possible to use the same extruder. DSC results still showed a denaturation enthalpy of 12.6 J g^{-1} compared to 61.8 J g^{-1} for untreated chromium shavings and 0 J g^{-1} for gelatin.

Pre-treatment allowed the use of a higher loading rate and increased the maximum daily methane production. The trial with untreated chromium shavings (Figure 4.14a) had to be stopped at a loading rate of 1.5 g kg^{-1} d^{-1} but the extruded shavings (Figure 4.14b) could be tested up to a loading rate of 1.9 g kg^{-1} d^{-1} and the shavings treated hydrothermally (Figure 4.14c) up to 2.0 g kg^{-1} d^{-1}. The untreated chromium shavings reached the maximum daily methane production, 0.41 L kg^{-1} d^{-1}, on the 31st day of digestion with a loading rate of 1.0 g kg^{-1} d^{-1}. The extruded shavings reached a higher value, 0.45 L kg^{-1} d^{-1}, on the 52nd day of digestion, but with a loading rate of 1.4 g kg^{-1} d^{-1}. Similarly, for the shavings treated hydrothermally, the maximum daily methane production was 0.45 L kg^{-1} d^{-1} on the 48th day of digestion with a loading rate of 1.5 g kg^{-1} d^{-1}.

The theoretical methane yield calculated using the basic elementary formula of bovine hide collagen and Equation 2.6 was found to be 393 L kg^{-1} (in organic dry matter of the substrate). Considering the total substrate added to each reactor it is possible to calculate how much of the maximum capacity of the substrate to produce methane was used. The trial using untreated chromium shavings as a substrate produced 70% of the substrate capacity added. The trial using extruded shavings produced 66% of the capacity. And finally, the trial using shavings treated hydrothermally produced 70% of the substrate capacity. Results are in accordance with literature were a conversion rate between 50 and 70% for proteins is expected (VDI 4630, 2006). It was found that the continuous process reached higher conversion rates for all substrates studied when compared with batch results. Agustini et al. (2018) studied the scale-up of anaerobic digestion of chromium shavings in batch tests and concluded that a larger scale can increase biogas yields.

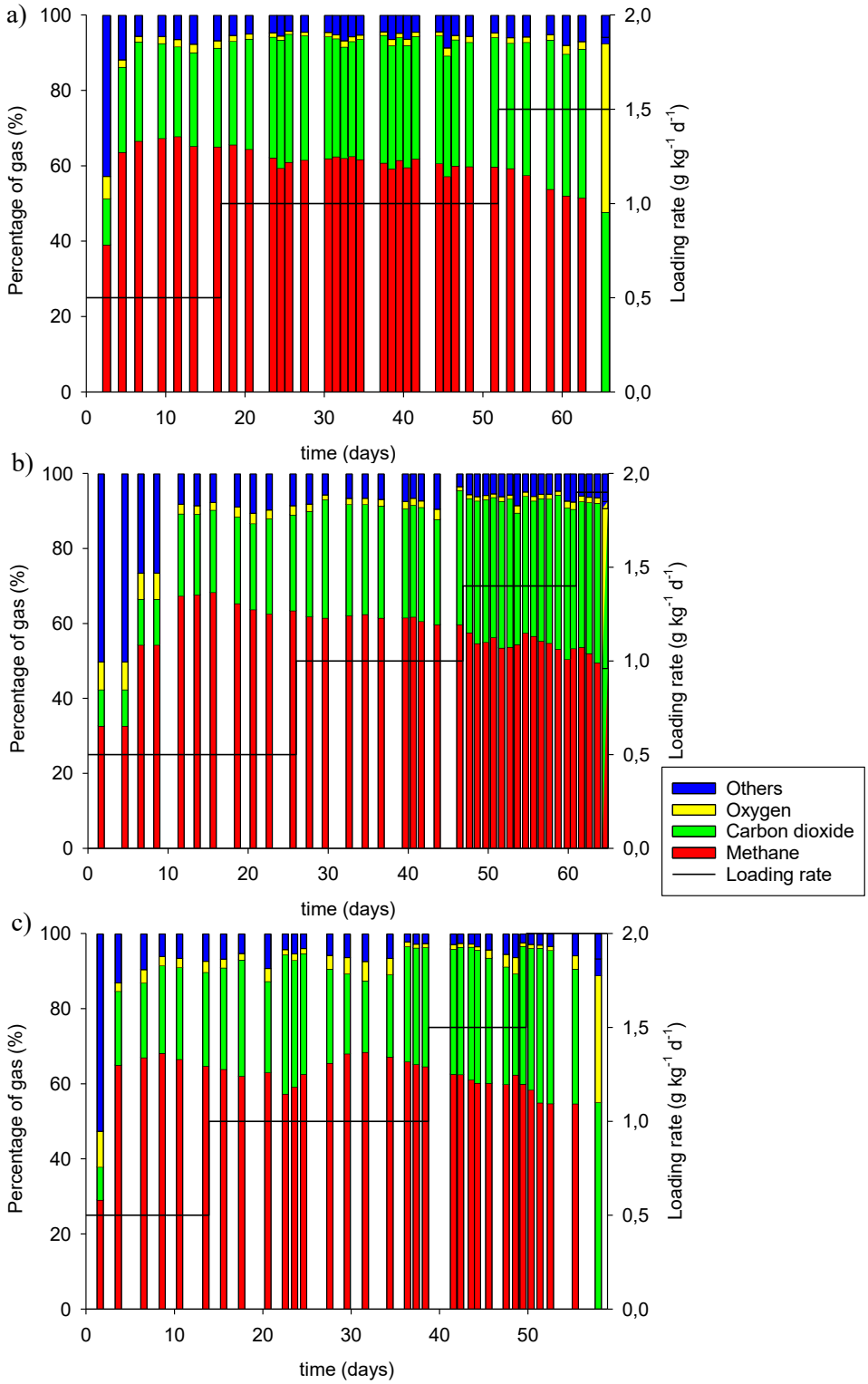

Fig. 4.15: *Quality of the biogas produced during digestion of chromium shavings (a), shavings extruded dry at 100 °C (b), and shavings treated hydrothermally at 140 and 150 °C (c).*

Figure 4.15 shows the quality of the biogas produced in the reactors. The production of methane was low at the beginning of digestion. The composition of the produced biogas showed less than 40% of methane until the third day of digestion for the reactor fed with untreated chromium shavings, until the fifth day for the extruded shavings reactor, and until the second day for the reactor using shavings treated hydrothermally. After that, methanation increases reaching its highest level of about 68% for the untreated chromium shavings, the extruded shavings, and the shavings treated hydrothermally. The reactor fed with extruded shavings showed the longest delay in methanation among the substrates studied. This was probably caused by the ingress of oxygen at the beginning of the anaerobic digestion trial.

A high level of oxygen in the produced biogas was registered for all substrates studied at the beginning of digestion, about 7%. This value drops during the first days of digestion as it is consumed by facultative anaerobic bacteria in the hydrolysis process. Due to the ingress of oxygen in the reactor fed with extruded shavings, oxygen only dropped after the ninth day. The presence of a small quantity of oxygen during digestion was verified for all continuous trials. This is probably oxygen from the change of gasbags and does not affect the anaerobic digestion.

The biogas quality was slightly reduced with every rise of the loading rate by 0.5 units. The only exception was the rise to a loading rate of 1.0 g kg^{-1} d^{-1} for the reactor fed with shavings treated hydrothermally. As previously seen, high loads of substrate can cause inhibition of methanogenesis and, in this case, this is reflected in the quality of the biogas produced.

Trials for all substrates showed a reduction of biogas quality for the loading rate at which they had their maximum daily methane production. Apart from that, the quality values were stable. It was possible to produce more biogas by increasing the load, however, methanogenesis started to be inhibited. At the end of anaerobic digestion, the quality dropped indicating a failure of digestion.

No studies covering the anaerobic digestion of chromium leather waste in continuous trials could be found for comparison. Some authors studied the digestion of tannery waste in continuous (López et al., 2015a and 2015b) or semi-

continuous mode (Berhe and Leta, 2018; Kameswari et al., 2015; Zupančič and Jemec, 2010) but the material was always collected from tanneries prior to the tanning step and this is known to be used already on industrial scale (Schuberth-Roth, 2013). These studies mainly used fleshings as substrate, which are also not suitable for comparison due to a high fat content.

4.2.2.2 Inhibition of digestion

In order to monitor the reactor stability and possible inhibitions, biomass samples were collected weekly and analyzed regarding their concentration of volatile fatty acids. Figure 4.16 shows the results for the three continuous trials. The error bars represent the standard deviation for the experimental data.

Volatile fatty acids are important intermediate products resulting from the acidogenesis phase during anaerobic digestion. However, they could cause inhibition and failure of the digester. To avoid failure the total concentration of volatile fatty acids should be lower than 4 g L^{-1}, the acetic acid concentration should be lower than 3 g L^{-1}, the isobutyric acid concentration lower than 0.5 g L^{-1}, and the propionic acid concentration lower than 1 g L^{-1} (Kaiser et al., 2008), but a concentration of propionic acid higher than 0.3 g L^{-1} is sufficient to disturb anaerobic digestion (Deublein and Steinhauser, 2008).

Once again the concentration of volatile fatty acids is consistent with that expected for the amino acids of bovine hide collagen fermented through Stickland reaction (Ramsay and Pullammanappallil, 2001). The key volatile fatty acid produced during the three trials was acetic acid, followed by isovaleric acid and propionic acid.

The reactor fed with untreated chromium shavings (Figure 4.16a) showed a stable total volatile fatty acid concentration for a loading rate of up to 1.0 g kg^{-1} d^{-1} but the concentration increased at a loading rate of 1.5 g kg^{-1} d^{-1} to more than 4 g L^{-1}, and the daily methane production dropped. Additionally, for the final sample collected the acetic acid concentration was 3.5 g L^{-1} and the propionic acid concentration reached 0.6 g L^{-1} indicating a complete failure of the reactor at this loading rate.

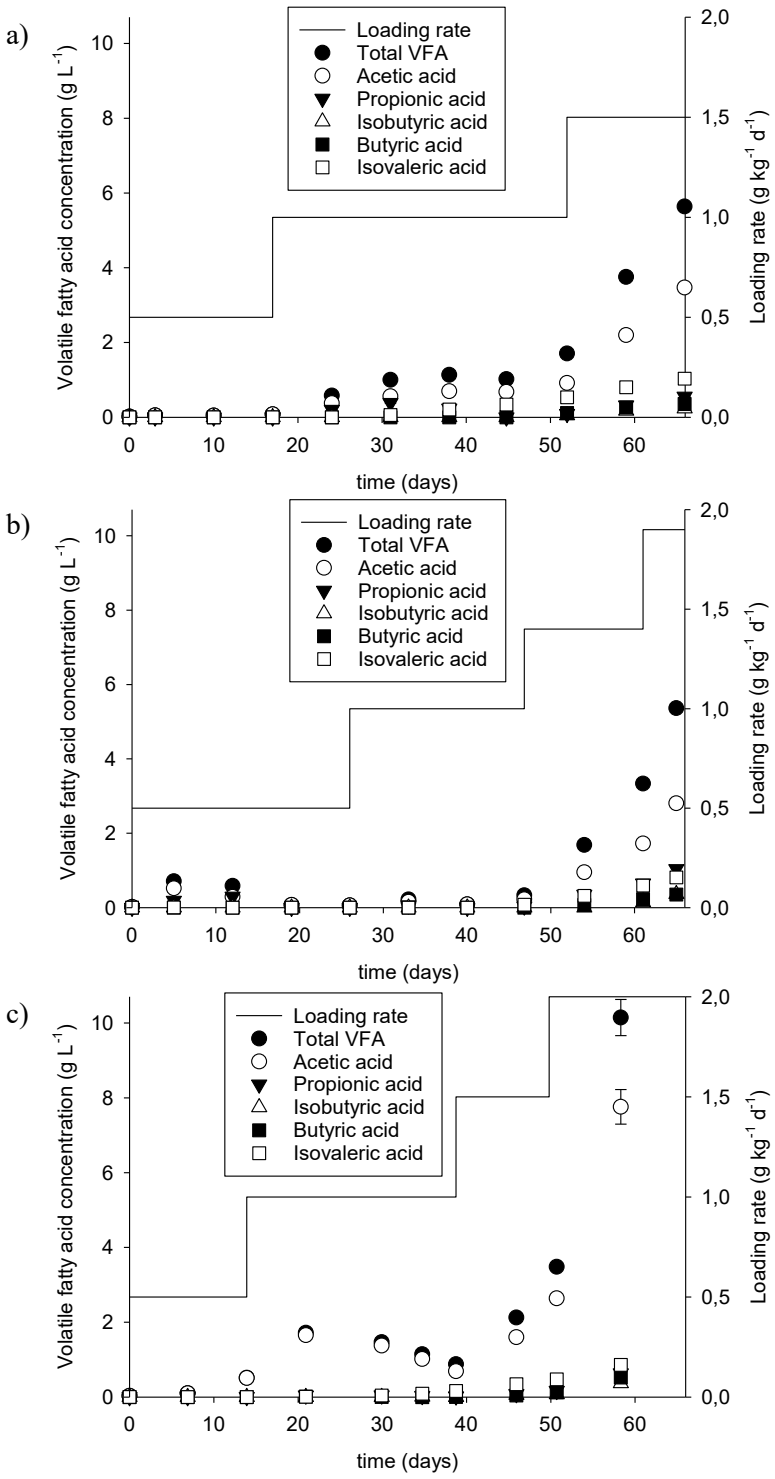

Fig. 4.16: *Volatile fatty acid concentrations during anaerobic digestion of untreated chromium shavings (a), shavings extruded dry at 100 °C (b), and shavings treated hydrothermally at 140 °C and 150 °C (c) (source: Gomes et al., 2019b).*

Similarly, using extruded shavings as substrate (Figure 4.16b), the total volatile fatty acid concentration was low for a loading rate of up to 1.0 g kg^{-1} d^{-1}. There was a small increase of acid concentration for the first loading rate corresponding with the ingress of oxygen registered for this reactor, but the system recovers its former stability and the acid concentration drops again. The acid concentration started increasing at a loading rate of 1.4 g kg^{-1} d^{-1} without reaching an inhibitory concentration, which could explain the unstable daily methane production values at this loading rate. Finally, for the last loading rate, the total acid concentration reached 5.4 g L^{-1} and the propionic acid concentration reached a value of 1 g L^{-1} indicating a failure of the reactor. The acetic acid and isobutyric acid concentrations were also high for this last biomass sample but without reaching an inhibition concentration.

The reactor fed with shavings treated hydrothermally (Figure 4.16c) also showed a small increase in the volatile fatty acid concentration due to the agitation failure at a loading rate of 1.0 g kg^{-1} d^{-1}. Again, the reactor recovers stability and the acid concentration drops. Otherwise, the reactor showed stable volatile fatty acid concentrations. The concentration only increased for the last biomass sample collected at a loading rate of 1.5 g kg^{-1} d^{-1}. Inhibition began at the last loading rate and daily methane production dropped. The total volatile fatty acid concentration increased to up to 10.1 g L^{-1}, the acetic acid concentration to up to 7.8 g L^{-1}, propionic acid to up to 0.6 g L^{-1}, and isobutyric acid to up to 0.4 g L^{-1}.

The chromium content in the reactors could inhibit the methanogenic archaea without affecting the acidogenic bacteria resulting in the accumulation of volatile fatty acids. Cr^{3+} ions could also have other negative effects on the anaerobic digestion such as a decrease in the total gas production rate, a fall in the pH value, a decrease in the percentage of methane in the produced biogas, or a decrease in the COD removal efficiency (Alkan et al., 1996). The pH value and the chromium content of the biomass samples collected were analysed. The results are shown in Table 4.12.

Tab. 4.12: *pH values and chromium content of the samples collected during digestion.*

Time (d)	Loading rate (g kg^{-1} d^{-1})	pH	Chromium (%)*
CS			
0	0.5	8.1	1.1
3			1.0 ± 0.0
10		7.9	1.4 ± 0.1
17		7.9	1.6 ± 0.1
24	1.0	7.9	1.9 ± 0.0
31		8.0	2.4 ± 0.1
38		8.1	2.7 ± 0.0
45		8.1	3.5 ± 0.0
52		8.1	3.3 ± 0.1
59	1.5	8.1	3.9 ± 0.1
66		8.0	4.3 ± 0.1
E100D			
0	0.5	8.3	1.0 ± 0.0
5		7.8	1.3 ± 0.1
12		7.9	1.6 ± 0.0
19		8.0	2.0 ± 0.1
26		7.9	2.5 ± 0.0
33	1.0	8.0	3.1 ± 0.1
40		8.1	3.5 ± 0.1
47		8.0	3.8 ± 0.1
54	1.4	8.0	4.7 ± 0.1
61		8.1	4.9 ± 0.4
65	1.9	8.0	4.5 ± 0.0
H140 - H150			
0	0.5	8.1	1.2 ± 0.0
7		8.0	1.6 ± 0.0
14		8.0	1.7 ± 0.0
21	1.0	7.8	2.3 ± 0.0
30		8.1	2.8 ± 0.1
35		8.1	3.1 ± 0.0
39		8.1	3.1 ± 0.0
46	1.5	8.1	3.5 ± 0.1
51		8.1	4.7 ± 0.0
58	2.0	8.0	4.7± 0.0

*Dry basis; mean ± standard deviation, n = 2; measured as chromium oxide

Source: Gomes et al., 2019b.

The pH value of the biomass during digestion was very stable, between 7.8 and 8.1, causing no disturbance in the system. The same stability was previously seen in the batch trials. It is known that a pH value between 5.2 and 6.3 is perfect for bacteria from the hydrolysis and acidogenesis phases, however these bacteria also work for a slightly higher pH value with slightly reduced activity. For the acetogenic bacteria and methanogenic archaea, the pH value needed ranges from 6.5 to 8 and, therefore, the pH value of the reactor must be within this range (FNR, 2012).

For Deublein and Steinhauser (2008) the toxic effect of Cr^{3+} as free ion starts at concentrations of 28 to 300 mg L^{-1}. Alkan et al. (1996) studied the presence of chromium during anaerobic digestion in depth, testing the direct injection of Cr^{3+} ions in anaerobic digesters. They concluded that a shock injection of only 500 mg L^{-1} of Cr^{3+} was necessary to lead to a system failure. However, a total concentration of up to 1140 mg L^{-1} of Cr^{3+} was tolerated in the anaerobic digester when injected in a stepwise manner. In the latter case, the soluble chromium concentration in the digester supernatant was measured on the following day of each injection and only 20 mg L^{-1} of Cr^{3+} were found. The remainder of the chromium was assumed to have precipitated in other forms, for which however, no measurements were made. This shows that methanogenic archaea can adapt to the presence of chromium and that not all of the chromium in the digester is soluble. In fact, soluble chromium is more toxic with regard to anaerobic digestion than insoluble chromium. Furthermore, the authors concluded that Cr^{3+} reduced the production of methane and increased the concentration of volatile fatty acids indicating that this metal has direct effect on the methanogenic bacteria.

For the continuous trials studied, chromium was present in the initial inoculum and it was added in a stepwise manner, as the substrates contain chromium. At the end of the trials, a total chromium content of almost 5% (measured as chromium oxide) was achieved in the biomass of all continuous trials which, as expected, is much higher than the values seen previously in the batch trials.

The chromium content of the anaerobic sludge used as inoculum was high because the sludge was generated in a tannery. The inoculum for the reactor fed

with untreated chromium shavings had a total chromium content of 324 mg L^{-1}, the inoculum for the extruded shavings reactor had a total chromium content of 233 mg L^{-1}, and the inoculum for the reactor fed with shavings treated hydrothermally had a content of 369 mg L^{-1}. The bacteria present in the initial sludge were already adapted to high quantities of chromium before its utilization as inoculum in the anaerobic digestion. The biomass of all continuous trials reached the concentration found to be toxic by Alkan et al. (1996). This is true for the loading rates determined to be more appropriate for each substrate that is 1.0 g kg^{-1} d^{-1} for the reactor fed with untreated chromium shavings, 1.4 g kg^{-1} d^{-1} for the reactor fed with extruded shavings, and 1.5 g kg^{-1} d^{-1} for the reactor fed with shavings treated hydrothermally. However, the soluble part of the total chromium in the reactors was not measured in this paper. Free ions of chromium are known to be more toxic to the methanogenic archaea than insoluble chromium.

This indicates that the total chromium concentration found in the reactors studied was not inhibitory for the methanogenic archaea. However, further studies should measure all fractions of chromium in the anaerobic reactor. Determination of a more suitable limit concentration of chromium would only be possible with a long-term continuous trial at an appropriate loading rate, without other inhibitions. This long-term trial should be carried out with the objective of reaching a maximum chromium content in the final biomass, which could ease the removal of chromium at the end of the process. Since chromium is normally obtained from mining at low cost, the average chromium content for which recycling is economically reasonable is between 14 to 40% by weight (Bertau, 2018). If this chromium content is reached and the chromium can be extracted from the biomass, it will be possible to use the removed chromium in the tanning step of the leather-making process.

A high concentration of hydrogen sulfide in the generated biogas can be extremely damaging to the process. Not only because of inhibitory effects but also because of its toxicity even at low concentrations, and corrosive effects which reduce equipment lifetime. The presence of sulfate could form hydrogen sulfide (Equation 2.5). Figure 4.17 shows the concentration of H_2S in the biogas produced and the concentration of sulfate in the biomass samples collected.

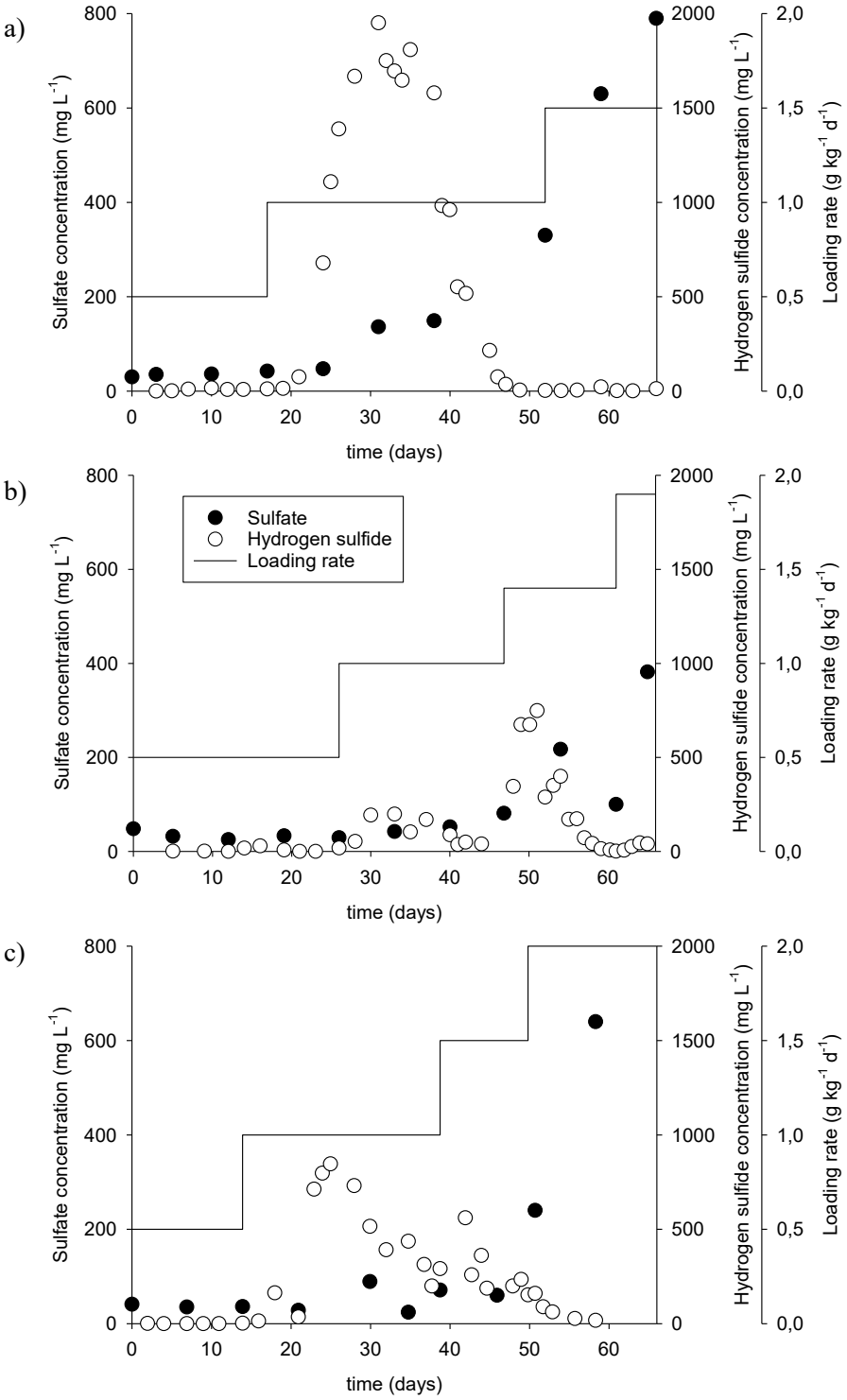

Fig. 4.17: *Concentration of total sulfate in the biomass collected and hydrogen sulfide in the biogas formed at different reaction times for the reactor fed with chromium shavings (a), shavings extruded dry at 100 °C (b), and shavings treated hydrothermally at 140 and 150 °C (c).*

The concentration of hydrogen sulfide in biogas depends on the substrate. Bovine hide collagen presents low quantities of sulfur in its basic elementary formula (The UniProt Consortium), which is not enough for the production of the registered quantities of H_2S. Furthermore, organic sulfur from protein is not completely transferred to the gas phase, part of it remains in the biomass (Polster and Brummack, 2005). H_2S is produced from the reduction of inorganic sulfate when sulfate-degrading bacteria compete with the methanogenic archaea to produce hydrogen sulfide. The inorganic sulfate present in the reactor originates from the basic chromium sulfate used in the tanning process to produce leather.

All reactors started producing hydrogen sulfide when the loading rate was raised to 1.0 g kg^{-1} d^{-1}. Afterwards, the increase in concentration of this toxic gas was controlled by adding iron chloride. The addition of iron chloride and the concentration of iron in the biomass samples collected can be seen in Figure 4.18. If H_2S drops, the concentration of sulfate steeply rises as H_2S can no longer be converted and the loading rate for the substrate is higher. The trial using untreated chromium shavings as substrate reached a high concentration of H_2S (Figure 4.17a) as it was the first trial executed and the addition of iron chloride was based on the author's own experience. However, the two continuous trials subsequently performed (Figures 4.17b and 4.17c) indicate that it is possible to easily control the H_2S concentration with the addition of iron chloride.

As previously seen, bovine hide collagen, the main component of the substrates studied, is a nitrogen-rich substrate with a C/N ratio of 3.1 and there is the possibility of inhibition by ammonia. The ammonia content in the biomass of the reactors was monitored during anaerobic digestion. Figure 4.19 shows the concentration of ammonia results. The sample point 0 represents the ammonia content of the inoculum before digestion.

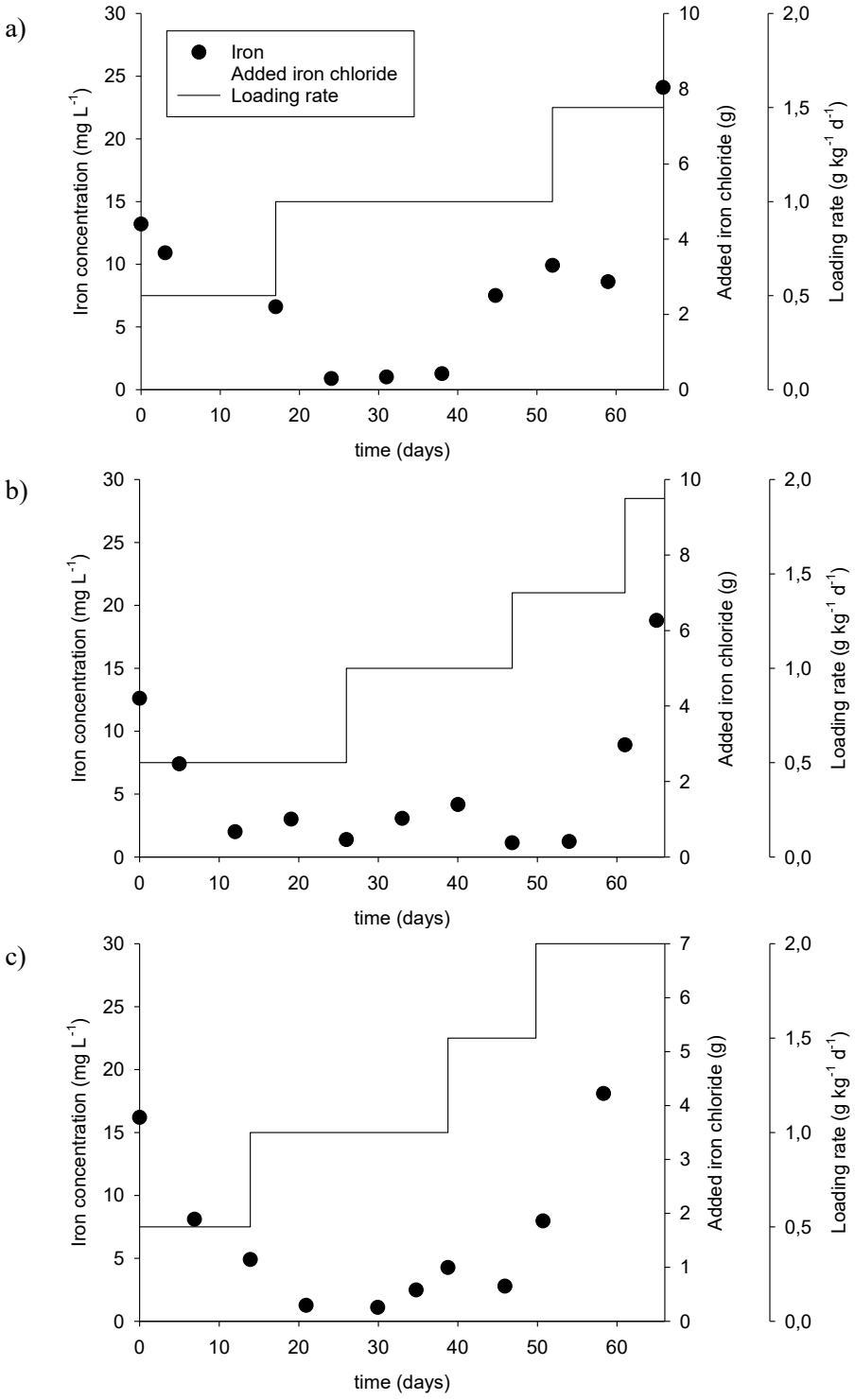

Fig. 4.18: *Concentration of iron in the biomass collected and mass of added iron chloride at different reaction times for the reactor fed with untreated chromium shavings (a), shavings extruded dry at 100 °C (b), and shavings treated hydrothermally at 140 °C and 150 °C (c).*

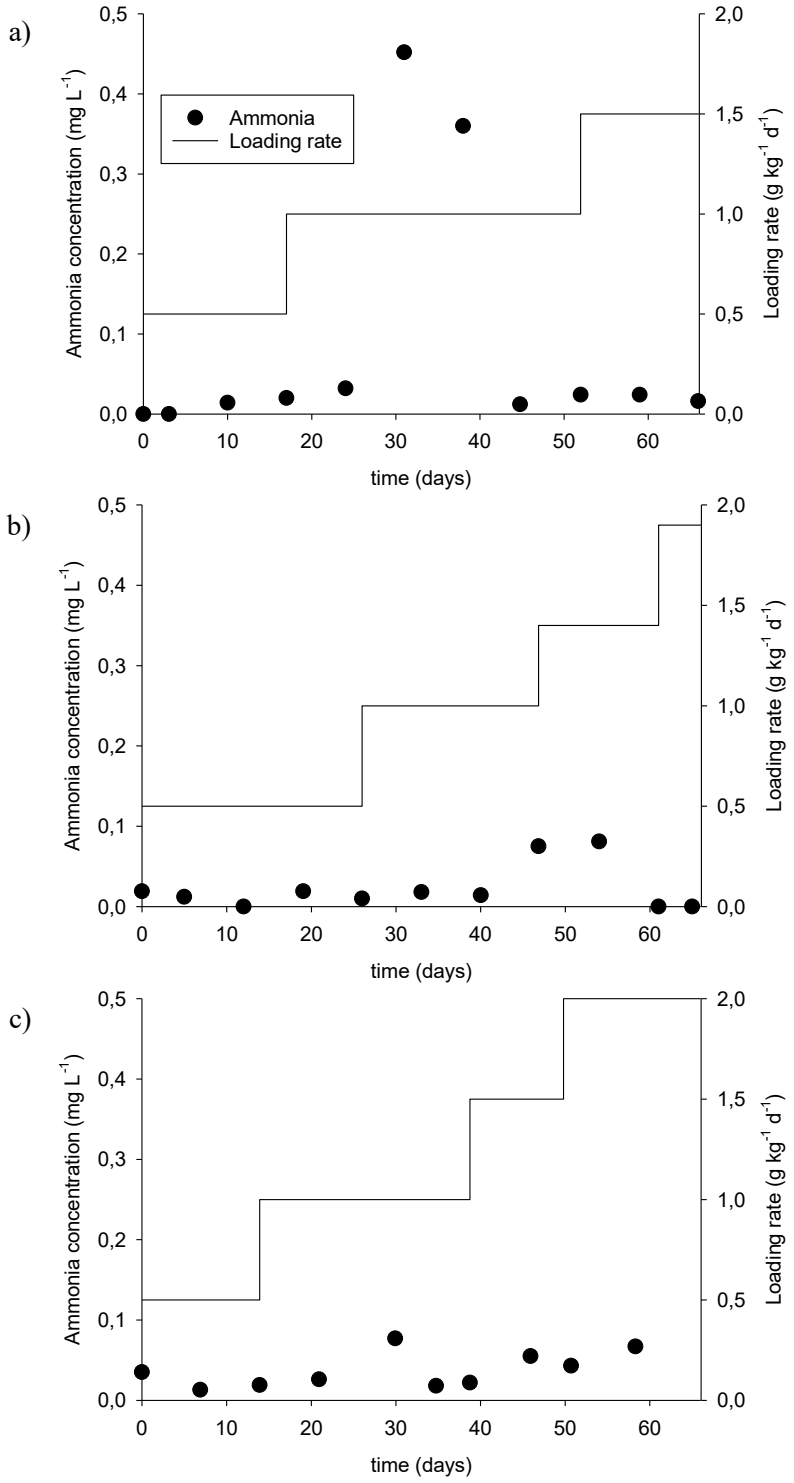

Fig. 4.19: *Concentration of ammonia found in the biomass collected at different reaction times for the reactor fed with untreated chromium shavings (a), shavings extruded dry at 100 °C (b), and shavings treated hydrothermally at 140 °C and 150 °C (c).*

The inhibition level of ammonia is known to be 1.5 to 3.0 g NH_4-N L^{-1} (Drosg et al., 2013). With regard to the three continuous trials, ammonia is present in the biomass in a concentration below 0.5 g NH_4-N L^{-1} and caused no problem for anaerobic digestion. The ammonia generated by fermentation of the amino acids from collagen did not accumulate, presumably as it was used for bacterial growth.

Table 4.13 shows an overview of the inhibitors present in the reactor and their inhibitory concentration. Literature values are compared to inhibitory concentrations found in the reactors at different loading rates.

Different sources of inhibition are present in the reactor for the substrates studied. The most important inhibitor appears to be the volatile fatty acids, which showed an inhibitory concentration for the two loading rates tested last in the three continuous trials. This could be the cause for the failure of the reactors. However, the accumulation of these acids was not sufficient to cause acidification of the biomass. The pH values are slightly above the optimum during digestion and there is a small reduction for the last loading rate.

For all continuous trials, the total chromium content was above inhibitory concentration regarding the second or third loading rate tested, and continues to increase. However, methane is produced effectively. The biomass appears to be adapted to the presence of chromium. Concentrations of hydrogen sulfide above the inhibitory concentration did not disturb anaerobic digestion but needed to be controlled with the addition of iron salts.

Tab. 4.13: *Inhibitors in anaerobic digestion and values reached in the continuous trials.*

Inhibitor	**Inhibitory value**	**Reference**
Volatile fatty acid	> 4 g L^{-1}	Kaiser et al. (2008)
pH	< 6.5 and >8	FNR (2012)
Total chromium	> 1.14 g L^{-1}	Alkan et al. (1996)
Hydrogen Sulfide	> 0.05 g L^{-1}	Deublein and Steinhauser (2008)
Ammonia	> 1.5 g L^{-1}	Drosg et al. (2013)
CS	**Value reached**	**Loading rate (g kg^{-1} d^{-1})**
Volatile fatty acid	5.6 g L^{-1}	1.5
pH	8.1	1.0, 1.5
Total chromium	1.4, 2.0 g L^{-1}	1.0, 1.5
Hydrogen Sulfide	2.0 g L^{-1}	1.0
Ammonia	-	-
E100D	**Value reached**	**Loading rate (g kg^{-1} d^{-1})**
Volatile fatty acid	5.4	1.9
pH	8.1	1.0, 1.4
Total chromium	1.2, 1.8, 1.7 g L^{-1}	1.0, 1.4. 1.9
Hydrogen Sulfide	0.2, 0.7 g L^{-1}	1.0, 1.4
Ammonia	-	-
H140-H150	**Value reached**	**Loading rate (g kg^{-1} d^{-1})**
Volatile fatty acid	10.1 g L^{-1}	2.0
pH	8.1	1.0, 1.5
Total chromium	1.8, 2.0 g L^{-1}	1.5, 2.0
Hydrogen Sulfide	0.8, 0.6, 0.09 g L^{-1}	1.0, 1.5, 2.0
Ammonia	-	-

4.2.2.3 Degradation of the substrate

Degradation of the substrate was evaluated analyzing the biomass samples collected weekly during anaerobic digestion. Table 4.14 shows the characterization of the biomass samples collected. The samples were analyzed regarding their collagen content, organic matter, and chromium content.

Tab. 4.14: *Biomass characterization of the samples collected during continuous digestion.*

Time (d)	Loading rate (g kg^{-1} d^{-1})	Collagen (%)*	Organic matter (%)*
CS			
0	0.5	2.9 ± 0.3	39.5 ± 1.4
3		4.3 ± 0.9	41.4 ± 1.2
10		4.4 ± 0.4	41.2 ± 1.8
17		5.8 ± 0.6	38.7 ± 0.2
24	1.0	6.3 ± 0.7	41.1 ± 0.3
31		5.1 ± 0.4	40.8 ± 0.0
38		3.6 ± 0.4	40.2 ± 0.3
45		2.5 ± 0.1	40.3 ± 0.2
52		2.3 ± 0.1	41.3 ± 0.2
59	1.5	2.6 ± 0.2	44.6 ± 0.3
66		3.3 ± 0.0	45.4 ± 0.1
E100D			
0	0.5	2.7 ± 0.3	38.3 ± 0.3
5		3.2 ± 0.1	38.4 ± 1.2
12		3.3 ± 0.1	38.5 ± 0.3
19		3.6 ± 0.1	39.6 ± 1.4
26		3.4 ± 0.1	37.9 ± 0.1
33	1.0	3.4 ± 0.3	40.2 ± 0.5
40		4.5 ± 0.2	40.3 ± 0.2
47		6.5 ± 0.3	42.0 ± 0.1
54	1.4	3.4 ± 0.1	43.7 ± 0.2
61		3.1 ± 0.1	44.4 ± 0.1
65	1.9	2.8 ± 0.0	46.9 ± 0.1
H140 - H150			
0	0.5	3.3 ± 0.0	37.4 ± 0.2
7		3.7 ± 0.0	39.1 ± 0.3
14		4.0 ± 0.1	37.9 ± 0.1
21	1.0	5.6 ± 0.0	40.7 ± 0.3
30		4.8 ± 0.3	39.8 ± 0.6
35		5.1 ± 0.1	39.8 ± 0.4
39		5.2 ± 0.1	40.6 ± 0.5
46	1.5	4.9 ± 0.1	42.3 ± 0.3
51		5.0 ± 0.0	45.3 ± 0.4
58	2.0	4.0 ± 0.1	48.0 ± 0.1

*Dry basis; mean ± standard deviation, n = 3

An increase in the organic matter content of the biomass collected would indicate accumulation of unprocessed substrate and, therefore, inefficiency of anaerobic digestion. As seen in Table 4.14, the reactor fed with untreated chromium shavings showed a constant value of organic matter content (about 40%) for most of the time. An increase to 45% was detected at the end of the trial at a loading rate of 1.5 g kg^{-1} d^{-1}, indicating that the reactor was overloaded. At this loading rate methane production was very low and the added substrate accumulated.

The reactor fed with the extruded shavings had an organic matter content of about 39% at a loading rate of 0.5 g kg^{-1} d^{-1}. At a loading rate of 1.0 g kg^{-1} d^{-1} the organic matter content remained constant at 40% and showed a slight increase to 42% at the end of this step. In a next step, an increase in organic matter to about 44% was observed at a loading rate of 1.4 g kg^{-1} d^{-1}. Finally, in the last step, the organic matter content increased to 47% at a loading rate of 1.9 g kg^{-1} d^{-1}.

For the reactor fed with shavings treated hydrothermally, the organic matter content of the biomass started at about 38% for the first loading rate. Proceeding to the next step, a loading rate of 1.0 g kg^{-1} d^{-1}, increased the organic matter content of the reactor to about 40%. The rise to a loading rate of 1.5 g kg^{-1} d^{-1} resulted in an increase to 45% and the last loading rate led to 48% of organic matter content, indicating accumulation.

At the end of digestion, the collagen content in the biomass was almost as low as it was at the beginning of the process for all reactors, showing that collagen was degraded and that there is no accumulation of collagen in the reactor. Therefore, the collagen of the added substrate was metabolized. However, the inorganic part of the substrates accumulated and the chromium content in the reactor increased.

Although a second batch of extruded shavings was used, which were not completely denatured, the second batch of the substrate could be degraded by anaerobic digestion. When the reactor was shut down, it contained 33.7 g of collagen (dry basis), which represents a final degradation of 96.5% of all of the col-

lagen added (953.8 g of collagen added). The reactor fed with untreated chromium shavings contained 45.6 g of collagen (dry basis) at shutdown, corresponding to 95.5% of degradation (1014.4 g of collagen added) and the reactor fed with shavings treated hydrothermally showed a final amount of collagen of 58.3 g (dry basis) meaning 95.3% of degradation regarding the collagen added (1233.4 g of collagen added).

Degradation of collagen after one feed was further studied for the reactor fed with shavings treated hydrothermally. After feeding the reactor with substrate on the 51st day of digestion at a loading rate of 2.0 g kg^{-1} d^{-1} small biomass samples were collected from the reactor at different times over a period of two days for collagen content analyses in triplicate. Figure 4.20 shows the collagen content results for the biomass samples collected. The error bars represent the standard deviation for the experimental data.

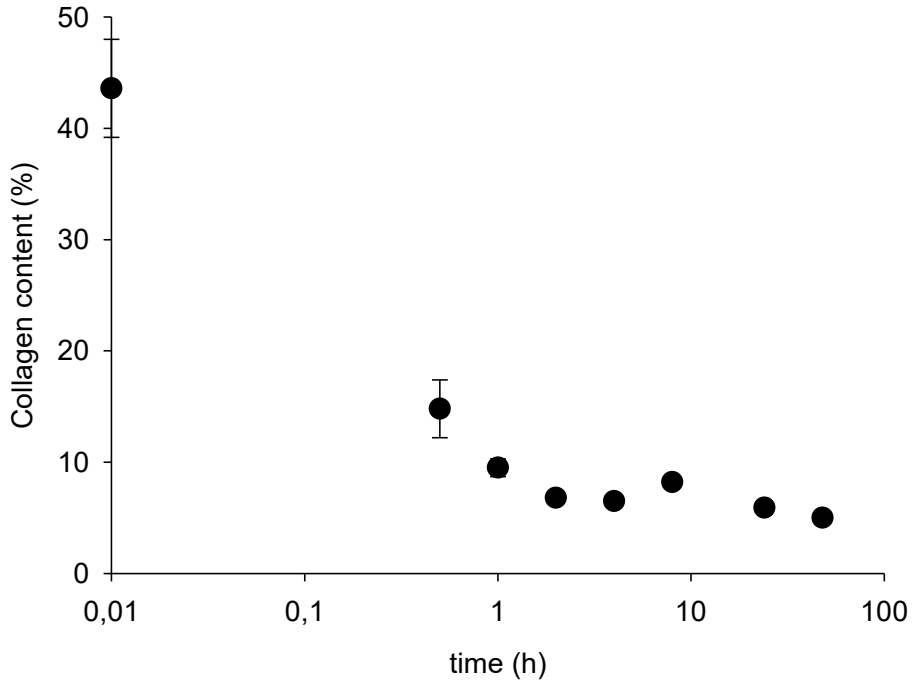

Fig. 4.20: *Collagen content of the biomass samples collected from the reactor fed with shavings treated hydrothermally after substrate feeding (logarithmic timeline).*

Prior to feeding, the reactor showed a collagen content of 5%. Shortly after feeding, the collagen content rose to about 44%, a very high value probably due to the inhomogeneity of the substrate, which can be seen in the high variance of the results. The substrate needed more time to be solubilized in the biomass. After two hours only, the collagen content in the biomass was almost as low as prior to substrate feeding, indicating that most of the collagen had been rapidly degraded. After forty eight hours, the collagen content dropped to 5%, the same collagen content detected prior to feeding. This leads to the conclusion that collagen was rapidly degraded at this loading rate even though organic matter accumulated (Table 4.14).

Although the detectable hydroxyproline in the biomass is low and a high content of collagen is rapidly degraded, degradation of all of the organic matter was not possible. Part of the substrate remains unprocessed in the biomass which can be seen in the organic matter content formed from organics of the inoculum and intermediate products of the hydrolyzed substrate, such as volatile fatty acids, amino acids, and peptides. In a previous paper (Gomes et al., 2017), it was shown that anaerobic digestion of inoculum without adding substrate leads to a final biomass of about 40% of organic matter content from which the conclusion can be drawn that part of the initial organic matter will remain unprocessed. The increase in organic matter content during digestion is a consequence of accumulation of unprocessed substrate in the form of intermediate products, which are not transformed into biogas. A possible reason for the accumulation of unprocessed organic matter is the inhibition of the anaerobic bacteria.

The substrate degradation was evaluated through a COD balance calculated with Equation 3.6 and the theoretical COD of bovine hide collagen as substrate (1.124 g_{O2} g^{-1}). The balance was made using the cumulative volume of methane produced in the reactors and the cumulative mass of added substrate starting on the first day of digestion (Figure 4.21). The cumulative COD degree of degradation shows degradation of substrate in the process as a whole and indicates that almost all of the substrate was successfully degraded. Furthermore, the biomass samples collected were analyzed regarding COD as another indicative of organics in the biomass. The results are shown in Figure 4.21.

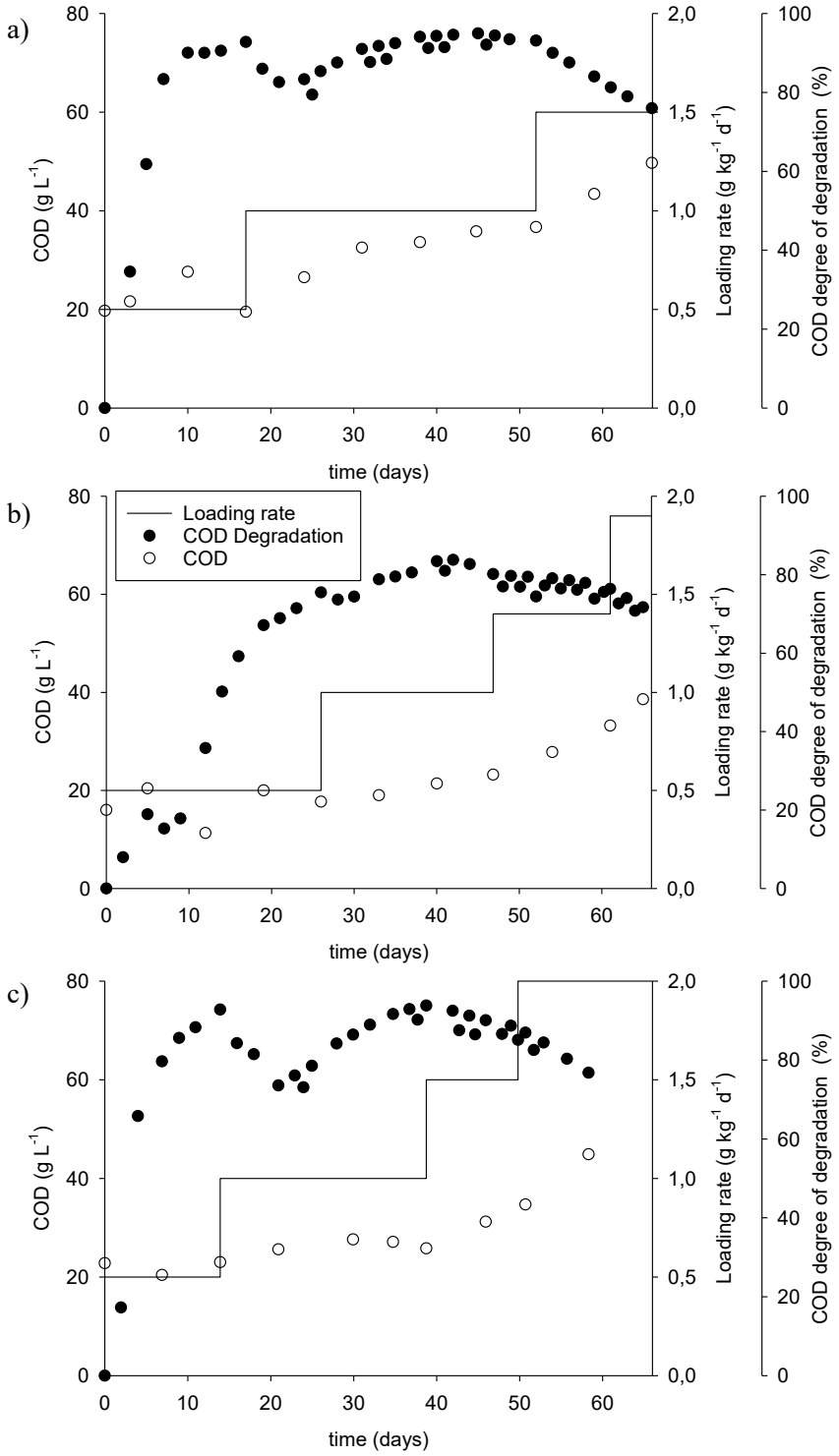

Fig. 4.21: *Cumulative COD degree of degradation during digestion and COD in the biomass samples collected at different reaction times for the reactor fed with chromium shavings (a), shavings extruded dry at 100 °C (b) and shavings treated hydrothermally at 140 and 150 °C (c).*

The maximum cumulative COD degree of degradation for the trial using untreated chromium shavings as substrate (Figure 4.21a) was 94.9% at a loading rate of 1.0 g kg^{-1} d^{-1}, the same loading rate that showed the highest daily methane production for this substrate. For this loading rate the cumulative COD degree of degradation at first reduces and later increases, indicating that the system needs to adapt to higher loads before starting production at its maximum capacity. For the same loading rate, COD increases slowly. In the last loading rate step, COD rises steeply and the cumulative COD degree of degradation declines.

The cumulative COD degree of degradation for the reactor fed with extruded shavings (Figure 4.21b) was affected by the ingress of oxygen on the fifth day of digestion. This trial takes longer than the other continuous trials to reach high COD degrees of degradation. The highest COD degree of degradation of 83.8% was achieved at a loading rate of 1.0 g kg^{-1} d^{-1} and not for the next step as expected, when the maximum daily methane production was reached. The value was lower than that for the other reactors. This is probably due to the instability caused by the increasing volatile fatty acids recorded for a loading rate of 1.4 g kg^{-1} d^{-1}. COD values started increasing at a loading rate of 1.0 g kg^{-1} d^{-1} and they became steeper for every subsequent loading rate step.

The reactor fed with shavings treated hydrothermally (Figure 4.21c) showed a maximum cumulative COD degree of degradation of 92.5% at a loading rate of 1.5 g kg^{-1} d^{-1}, the loading rate for the maximum daily methane production reached in this trial. There are two different reasons for the drop in cumulative COD degree of degradation at a loading rate of 1.0 g kg^{-1} d^{-1}. The system needed to adapt to a new loading rate after the rise from 0.5 g kg^{-1} d^{-1} to 1.0 g kg^{-1} d^{-1}, which caused a slight drop in degradation. Moreover, the agitation failure for this reactor on the 21st day intensified the drop. The COD values in the biomass samples were stable for a loading rate of up to 1.0 g kg^{-1} d^{-1}, increased slightly for the next step, and steeply at the last step.

Considering that the highest daily methane production for the untreated shavings was reached at a loading rate of 1.0 g kg^{-1} d^{-1}, it is possible to conclude that this is the most appropriate loading rate for these collagen-based materials. For

the reactor fed with extruded shavings the highest daily methane production was reached at a loading rate of 1.4 g kg^{-1} d^{-1} proving to be the most suitable loading rate for the extruded shavings among the loading rates studied. Finally, the reactor fed with shavings treated hydrothermally reached its highest daily methane production at a loading rate of 1.5 g kg^{-1} d^{-1}, indicating that this rate should be aimed at. Nevertheless, long-term trials of one year or more should prove this observation.

Accumulation of organic matter and the increasing concentrations of volatile fatty acids verified in the biomass samples collected for the last loading rate tested in all continuous trials will lead to the same conclusion. Pre-treatment of the chromium shavings allowed an increase of the loading rate of 40 to 50% and an increase of the daily methane production of almost 10%. A higher loading rate leads to a more economical process and it can be expected that the loading rate could be increased by 40 to 50% by pre-treating the chromium shavings. In contrast, the reactor volume for a given loading rate could be decreased. This increases the feasibility of digesting chromium shavings to produce biogas and energy in tanneries.

Additionally, the untreated chromium shavings can also be digested in continuous systems to produce methane but using a lower loading rate. An explanation for this observation is still missing, since this contradicts the common knowledge that untreated chromium leather cannot be digested in biogas reactors. The cause could be the degradation of the untreated material by several enzymes working simultaneously but more research is needed in this subject.

Results presented in this section indicate the loading rate to be aimed at when working with each of the substrates studied. However, it is important to bear in mind that these are preliminary results and long-term trials should be carried out testing the stability of anaerobic digestion at the aimed loading rates.

5 Conclusions

Chromium leather waste produced in tanneries is not commonly considered as substrate for biogas production through anaerobic digestion due to its high stability and chromium content. Nonetheless, this waste is produced in high quantities worldwide and the use as substrate would be a convenient solution to its disposal with the advantage of generating energy. To make this process feasible, pre-treatments can be used. This approach to produce biogas in batch and continuous scale has not been studied to date.

Three different pre-treatments were carried out to denature the stable collagen structure of chromium shavings and one pre-treatment was performed for leather offcuts. The aim was to enhance anaerobic digestion when using chromium leather waste as substrate to produce biogas. The hydrothermal and autoclaving pre-treatments increased degradation of chromium shavings by trypsin from 7% to more than 90% and extrusion to 35%. Extruded offcuts had a lower degradation by trypsin than chromium shavings due to their complexity acquired in the wet end and finishing process after tanning. Degradation of the chromium shavings using collagenase could also be increased from 12% to 86% by extrusion. To measure digestibility, it was possible to reach a high degradation degree by trypsin and collagenase of chromium shavings when using a pre-treatment.

Differential scanning calorimetry results showed that the hydrogen bonds that stabilize the collagen structure of the chromium leather waste against enzymatic degradation were completely or almost completely broken down for most of the pre-treated samples. An exception were shavings autoclaved for only three minutes due to insufficient pre-treatment time. Therefore, evaluation of the pre-treated materials showed that almost all of them were denatured. A

quantity of 4% of chromium measured as chromium oxide in chromium leather waste appears not to be toxic for anaerobic bacteria.

In biogas production batch tests, the trials with agitation were proven to have a better performance for the pre-treated substrates tested, facilitating the mass transfer inside the reactors. The pre-treatments were important to reduce the hydrolysis step of anaerobic digestion. Using extrusion as pre-treatment caused a decrease of the lag-phase at the beginning of the biogas production of the chromium shavings by four days and hydrothermal treatment resulted in a decrease of four to five days. Biogas production could be initiated already one day after starting the trials with shavings treated hydrothermally at 170 °C as substrate, the same lag-phase found for gelatin. Using a pre-treatment, the collagen of the chromium shavings degraded more intensively than without pre-treatment and it was possible to reach a collagen degree of degradation of above 98%.

Pre-treated chromium shavings showed a behaviour more similar to the partly hydrolyzed collagen gelatin than to untreated chromium shavings, confirming that the use of a pre-treatment on the substrate prior to digestion improves the biogas production. However, pre-treated shavings could not reach the biogas formation potential of gelatin. When chromium shavings are denatured by means of a pre-treatment, the hydrogen bonds, which stabilize the triple helix, are broken down but the structure is still arranged in long chains needing more time to degrade. Even gelatin with hydrolyzed short chains could not reach the biogas formation potential of other proteins due to its arrangement. Collagen yields less biogas than other proteins because its structure, denatured or not, is hard to degrade.

Even though there is a lag-phase before starting production, trials using untreated chromium shavings as substrate reached a similar biogas formation potential compared to pre-treated chromium shavings. Probably a combination of several enzymes present in the medium needs to work simultaneously to degrade the substrate but the enzymes remain to be identified. The use of extruded offcuts as substrate hindered biogas production compared to the use of

untreated chromium shavings. The wet end and finishing process in the automotive leather industry increases the complexity of the substrate for anaerobic digestion by adding organic matter to the substrate, which cannot be processed during anaerobic digestion. The use of different substrate to inoculum ratios (S/I) showed that in a bioreactor for all pre-treated and untreated substrates the ratio of 0.5 should be aimed at. Additionally, for trials using gelatin, extruded shavings, and extruded offcuts a ratio higher than 0.5 generated a two-phase decomposition. This kind of cumulative biogas production curve is known as diauxie.

As denatured collagen-based material is quickly hydrolyzed, the use of an S/I ratio higher than 0.5 will produce intermediate products with a velocity which the methanogenesis cannot follow. Analysis of the volatile fatty acid concentration of biomass samples during anaerobic digestion with an S/I ratio of 1.5 revealed that there was an excess of intermediate products during the biogas production plateau. When using pre-treated substrates, hydrolysis occurs quickly and the quantity of volatile fatty acids produced is high during acidogenesis. In these cases, the rate-limiting step in the anaerobic digestion is methanogenesis. In contrast, when the untreated chromium shavings were tested, hydrolysis needed more time for digestion and methanogenesis occurred slowly and without inhibitions. In this case, hydrolysis is the rate-limiting step.

The diauxie behaviour verified for pre-treated substrates with an S/I ratio higher than 0.5 was not caused by the high volatile fatty acid concentration during the biogas production plateau. As a consequence of inhibition of the methanogenic archaea, high concentrations of these unprocessed acids, mainly acetic acid and propionic acid accumulate. Further research should be carried out to determine the exact inhibitor or inhibitors of the methanogenic archaea in this case.

In continuous tests of biogas production, the reactors fed with pre-treated shavings showed better results than those using untreated chromium shavings as substrate, even with some technical problems during anaerobic digestion. The continuous biogas trials showed that it was possible to use a substrate loading rate which was 40 to 50% higher. Moreover, the daily methane production

could be increased by almost 10% by using pre-treated shavings rather than untreated chromium shavings as substrate.

Volatile fatty acids and organics in the biomass started to accumulate when the loading rate tested was too high for the system, leading to a lower daily methane production and failure of the reactor. Even at high loading rates, the added collagen was rapidly degraded. Most of it was degraded within two hours avoiding accumulation of unprocessed collagen in the reactor. The remaining organic matter at the end of the process is formed from intermediate products which could not be identified and were not transformed into biogas.

When using chromium leather waste as substrate, there is a potential of producing biogas with high concentrations of hydrogen sulfide mainly due to the basic chromium sulfate used in the tanning process. This could cause inhibition of methanogenesis, problems with equipment, and hazard to personnel. However, the concentrations of H_2S were easily controlled with the addition of iron chloride, a common H_2S scavenger.

The use of pre-treated chromium leather waste to produce biogas is a promising method to be performed in an industrial environment. An economical evaluation should be performed to verify if the pre-treatment costs are compensated by the energy gains. However, the reduction of the final waste, the disposal of which otherwise would generate costs, makes this method very attractive for industry purposes.

The reuse of chromium leather waste (collagen-based material) as a substrate in the biogas production is an interesting alternative to deal with the large amounts that are produced every day in tanneries. However, its degradation was particularly difficult compared to other proteins, such as casein or blood. An explanation for this could be that the collagen arrangement of triple helical chains needs considerable effort to be degraded. This could be investigated in future by analyzing intermediates, not only volatile fatty acids but also amino acids or oligo-peptides, which can result from degradation processes.

There are several theories about the reasons for diauxie. Results presented in this study lead to the conclusion that diauxie is not a direct consequence of volatile fatty acid accumulation as discussed by other authors. In the future studies should be carried out to determine the inhibitor of methanogenesis and the cause of diauxie behaviour.

Using leather offcuts to produce biogas appears to be difficult. Even though they were completely denatured by extrusion, the biogas formation potential was low and the remaining organic matter in the final biomass was higher than for the other substrates studied. For the substrate meant in the present paper, it is important to identify the unprocessed organic matter in the final biomass, which mainly results from the wet end and finishing process in the leather industry. Subsequently, a more appropriated pre-treatment should be studied and tested to break down these organics and increase their susceptibility to anaerobic digestion.

The unprocessed organic matter remaining in the final biomass in batch and continuous reactors is another challenge. These organics have been seen in final biomass coming from all of the substrates studied, including gelatin. All organic matter has a potential to be transformed into energy in anaerobic digestion. Consequently, this can be seen as a waste of resources. Furthermore, the final biomass is not suitable to be used as a fertilizer because of its high chromium content and its disposal would generate costs, which make the process less economically attractive. These organics should be further analyzed and identified to find a possible solution. If possible, a different pre-treatment, which allows these organics to be transformed into biogas, should be identified.

Long-term trials of one year or more should be carried out to confirm results obtained with continuous trials. The loading rate for each substrate aimed at should be tested to confirm if the conditions are stable over a long period of time and if there is accumulation of organic matter. Another important aspect is the accumulation of inorganic matter, mainly chromium. A chromium content of almost 5% was not toxic for the continuous reactors but a long-term trial could determine the maximum chromium content tolerated by the system. If a

higher chromium content was reached, extraction and recycling of the chromium could be reasonable and there could be further research in this regard.

Results hitherto obtained show that the pre-treatments can increase the efficiency of the biogas production from chromium leather waste (collagen-based) materials. The process could be accelerated, the waste destruction enhanced and the load of substrate increased. For batch reactors, the lag-phase is shorter and reduction of waste is more efficient. For continuous systems, daily methane productivity is higher, and the load of substrate can be increased or the reactor volume reduced. All these improvements increase the suitability of producing biogas using chromium leather waste as substrate in the industry.

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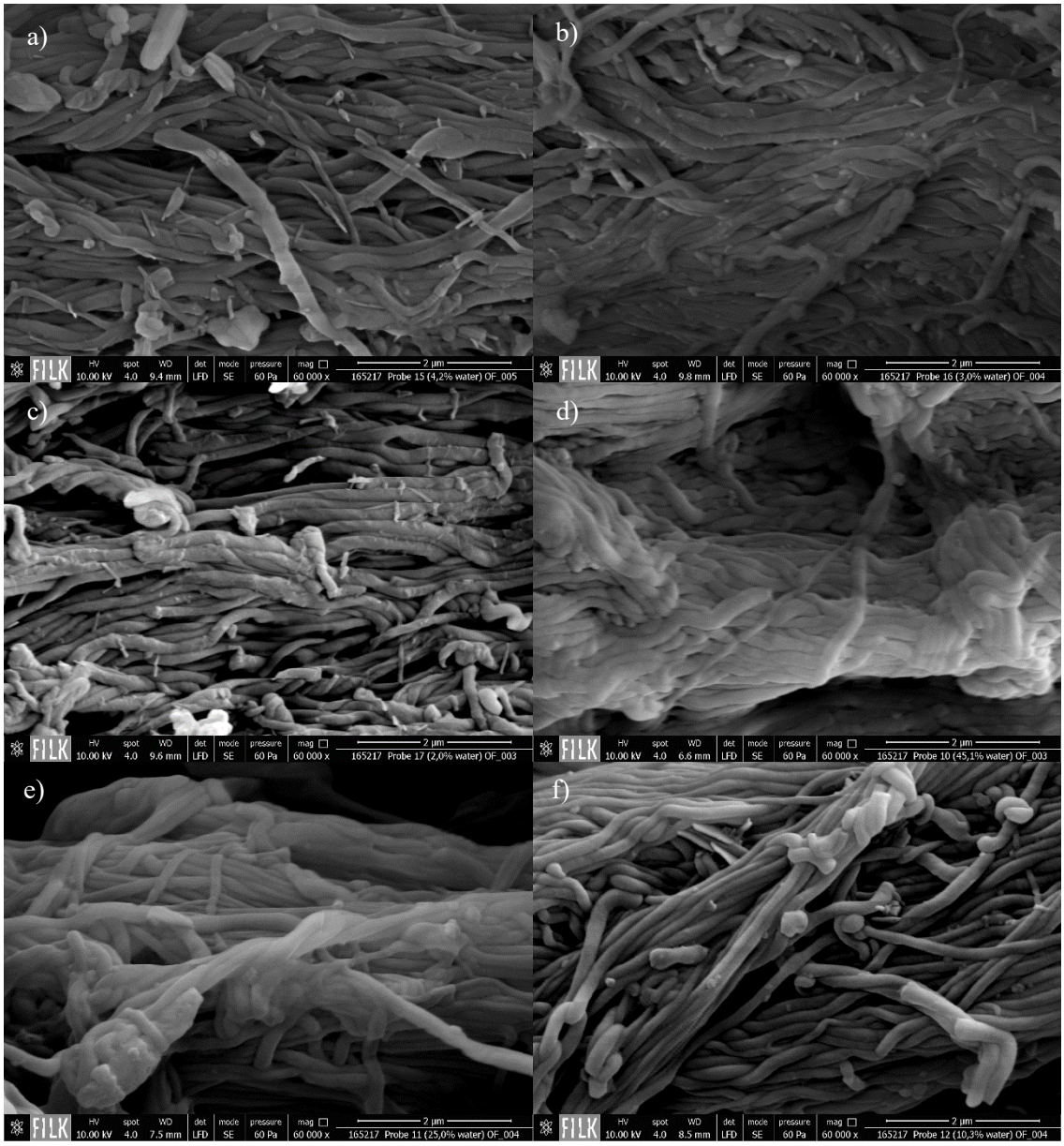
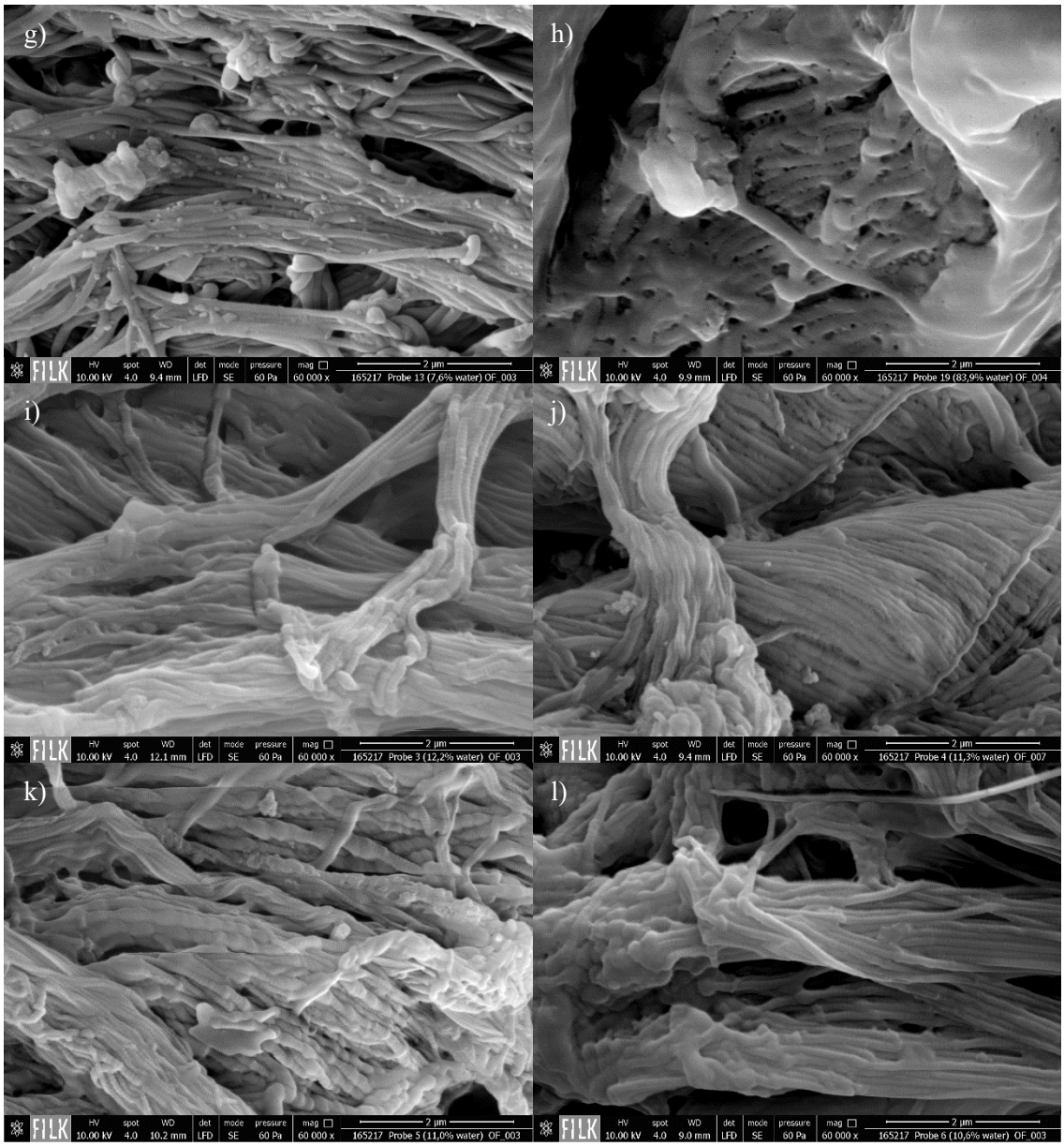
Appendix


a)
FILK HV 10.00 kV spot 4.0 WD 9.4 mm det LFD mode SE pressure 60 Pa mag 60 000 x 2 µm 165217 Probe 15 (4,2% water) OF_005
b)
FILK HV 10.00 kV spot 4.0 WD 9.8 mm det LFD mode SE pressure 60 Pa mag 60 000 x 2 µm 165217 Probe 16 (3,0% water) OF_004
c)
FILK HV 10.00 kV spot 4.0 WD 9.6 mm det LFD mode SE pressure 60 Pa mag 60 000 x 2 µm 165217 Probe 17 (2,0% water) OF_003
d)
FILK HV 10.00 kV spot 4.0 WD 6.6 mm det LFD mode SE pressure 60 Pa mag 60 000 x 2 µm 165217 Probe 10 (45,1% water) OF_003
e)
FILK HV 10.00 kV spot 4.0 WD 7.5 mm det LFD mode SE pressure 60 Pa mag 60 000 x 2 µm 165217 Probe 11 (25,0% water) OF_004
f)
FILK HV 10.00 kV spot 4.0 WD 8.5 mm det LFD mode SE pressure 60 Pa mag 60 000 x 2 µm 165217 Probe 12 (10,3% water) OF_004


g)
h)
i)
j)
k)
l)
FILK
HV spot WD det mode pressure mag 2 µm
10.00 kV 4.0 9.4 mm LFD SE 60 Pa 60 000 x 165217 Probe 13 (7,6% water) OF_003
10.00 kV 4.0 9.9 mm LFD SE 60 Pa 60 000 x 165217 Probe 19 (83,9% water) OF_004
10.00 kV 4.0 12.1 mm LFD SE 60 Pa 60 000 x 165217 Probe 3 (12,2% water) OF_003
10.00 kV 4.0 9.4 mm LFD SE 60 Pa 60 000 x 165217 Probe 4 (11,3% water) OF_007
10.00 kV 4.0 10.2 mm LFD SE 60 Pa 60 000 x 165217 Probe 5 (11,0% water) OF_003
10.00 kV 4.0 9.0 mm LFD SE 60 Pa 60 000 x 165217 Probe 6 (10,6% water) OF_003

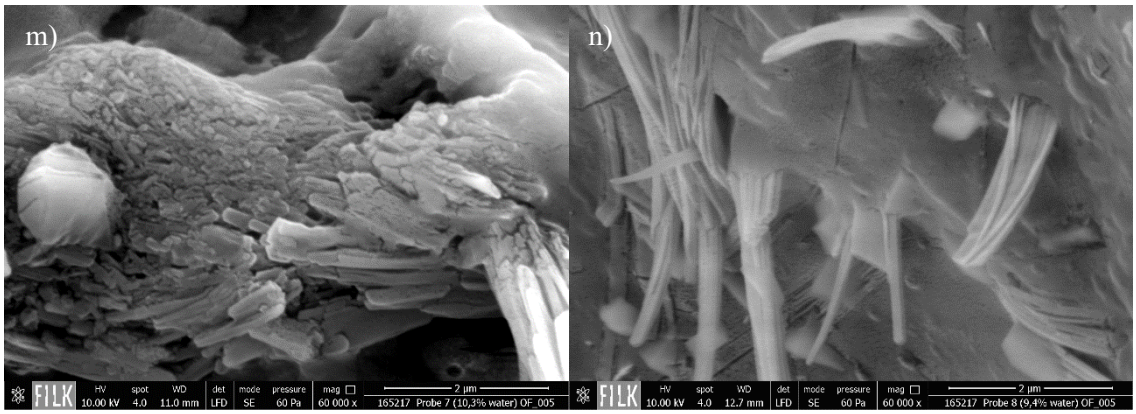


Fig. 5.1: *SEM images of shavings extruded dry at 130 °C (a), shavings extruded dry at 150 °C (b), shavings extruded dry at 170 °C (c), shavings extruded wet at 100 °C (d), shavings extruded wet at 130 °C (e), shavings extruded wet at 150 °C (f), shavings extruded wet at 170 °C (g), shavings treated hydrothermally at 150 °C (h), shavings autoclaved over a period of six minutes (i), shavings autoclaved over a period of twelve minutes (j), and shavings autoclaved over a period of 24 minutes (k), and shavings autoclaved over a period of 48 minutes (l), and shavings autoclaved over a period of 96 minutes (m), and shavings autoclaved over a period of 192 minutes (n) at 60,000 × magnification.*